Postsynaptic Current Mediated by Metabotropic Glutamate Receptors in Cerebellar Purkinje Cells

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INTRODUCTION

Synaptic signaling at glutamatergic synapses involves two main classes of receptors: ionotropic (iGluRs) and metabotropic glutamate receptors (mGluRs). Purkinje cells of the cerebellar cortex receive two types of glutamatergic fibers: a single climbing fiber and many thousands of parallel fibers. Both are known to activate iGluRs of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors and a slower component mediated by metabotropic glutamate receptors (mGluRs). The mGluR-mediated excitatory postsynaptic current (mGluR-EPSC) is evoked selectively by parallel fiber stimulation; climbing fiber stimulation is ineffective. The mGluR-EPSC is elicited most effectively with increasing frequencies of parallel fiber stimulation, from a threshold of 10 Hz to a maximum response at ~100 Hz. The amplitude of the mGluR-EPSC is a linear function of the number of stimulus pulses without any apparent saturation, even with >10 pulses. Thus mGluRs at the parallel fiber-Purkinje cell synapse can function as linear detectors of the number of spikes in a burst of activity in parallel fibers. The mGluR-EPSC is present from postnatal day 15 and persists into adulthood. It is inhibited by the generic mGluR antagonist (RS)-α-methyl-4-carboxyphenylglycine and by the group I mGluR antagonist (RS)-1-aminoindan-1,5-dicarboxylic acid at a concentration selective for mGluR1. Although the intracellular transduction pathway involves a G protein, the putative mediators of mGluR1 (phospholipase C and protein kinase C) are not directly involved, indicating that the mGluR-EPSC studied here is mediated by a different and still unidentified second-messenger pathway. Heparin, a nonselective antagonist of inositol-trisphosphate (IP₃) receptors, has no significant effect on the mGluR-EPSC, suggesting that also IP₃ might not be required for the response. Buffering intracellular Ca²⁺ with a high concentration of bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid partially inhibits the mGluR-EPSC, indicating that Ca²⁺ is not directly responsible for the response but that resting Ca²⁺ levels exert a tonic potentiating effect on the mGluR-EPSC.
when recorded by intracellular microelectrodes (Batchelor and Garthwaite 1997). This constitutes a saturation of the response with bursts of parallel fiber activity in the physiological range. Voltage-clamp recordings would help to distinguish which properties of the response are mediated by the mGluR and which are due to an involvement of voltage-activated conductances. In the present paper, whole cell patch-clamp recordings were used to throw more light on the input-output relationship of the synaptic signals mediated by mGluR in cerebellar Purkinje cells.

In addition, the role of \([Ca^{2+}]_i\) in the generation of the mGluR-EPSP remains a puzzle. An early hypothesis about the mechanism responsible for the current evoked by mGluR agonists pointed to an electrogenic Na\(^+\)/Ca\(^{2+}\) exchanger activated by an increase in \([Ca^{2+}]_i\) (Linden et al. 1994; Staub et al. 1992; Vranesic et al. 1991). Such a hypothesis has been challenged by the lack of \([Ca^{2+}]_i\) increases associated with the mGluR-EPSP (Batchelor et al. 1996). Moreover, the increase of \([Ca^{2+}]_i\) evoked by climbing fiber activity or by direct depolarization acts as a modulator of the mGluR-EPSP (Batchelor and Garthwaite 1997; Tempia et al. 1997). For this mechanism, it would be difficult to explain how the interaction of parallel and climbing fibers could be specific if \([Ca^{2+}]_i\) is used by both synapses to activate the mGluR-mediated response. Besides, the Na\(^+\)/Ca\(^{2+}\) exchanger hypothesis does not explain why the climbing fiber-evoked rise of \([Ca^{2+}]_i\), does not produce any mGluR-EPSP (Batchelor and Garthwaite 1997). For these reasons, it is of great importance to assess whether the only currently considered transduction mechanism, the phospholipase C (PLC) initiated cascade of second messengers resulting in the activation of protein kinase C (PKC) and in the release of Ca\(^{2+}\) from intracellular stores (see Pin and Duvousin 1995), really is involved in the production of the mGluR-mediated responses in Purkinje cells. A recent study in which mGluR was activated in cultured Purkinje cells by agonist applications (Netzeband et al. 1997) shows that the response, consisting in an increase in membrane excitability accompanied by an increase of \([Ca^{2+}]_i\), followed by a decrease in membrane excitability, is abolished by a PLC blocker. Our results describe for the first time in voltage clamp an excitatory postsynaptic current mediated by mGluR with excitatory postsynaptic current (mGluR-EPSC) and suggest that PLC and PKC are not involved in such response; some of the results have been presented in abstract form (Miniaci et al. 1996; Tempia et al. 1996b).

**Methods**

Experiments were performed on Wistar rats of either sex, 15- to 37-days old. The preparation of cerebellar slices was performed following a previously described technique (Edwards et al. 1989; Llinás and Sugimori 1980). Briefly, the animals were anesthetized either with halothane (Fluothane, Zeneca, Macclesfield, UK) or with CO\(_2\) and decapitated. The cerebellar vermis was removed rapidly and placed in ice-cold extracellular saline solution, which contained (in mM) 125 NaCl, 2.5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 1.25 NaH\(_2\)PO\(_4\), 26 NaHCO\(_3\), and 20 glucose and was bubbled with 95% O\(_2\)-5% CO\(_2\) so that the pH was 7.4. Parasagittal cerebellar slices (200-μm thick) were prepared using a vibratome (Vibrorslice 752, Campden Instruments, Loughborough, UK), kept for the first hour at 35°C and then at 25°C for the rest of the day. After the 1 h of incubation, a single slice was transferred to a recording chamber and continuously perfused at room temperature (22–26°C) with the saline solution bubbled with the 95% O\(_2\)-5% CO\(_2\) mixture. A Purkinje cell soma was visualized using a ×40 or a ×63 water-immersion objective of an upright microscope (Axioskop, Zeiss, Jena, Germany), and its upper surface was cleaned by gently blowing and sucking saline solution from a cleaning pipette (Edwards et al. 1989). The pipettes used for cleaning and for extracellular stimulation were pulled from sodalime glass to a tip diameter of 10–15 μm. For both cleaning and stimulation, the pipette was filled with extracellular saline solution. For stimulation, the pipette was pressed gently into the tissue, and negative voltage pulses ranging from 0.1 to 10 V with a duration of 100 μs were delivered. For patch-clamp recording, pipettes of borosilicate glass with a tip diameter of 2–3 μm were used. When filled with an intracellular solution the resistance was 1.4–2.5 MΩ. The internal pipette solution had one of the following compositions (in mM): 132 CsCl, 20 tetraethylammonium, 0.1 CaCl\(_2\), 2 MgCl\(_2\), 10 N\(_2\)-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 4 Na\(_2\)ATP, 0.4 Na\(_2\)guanosine 5'-triphosphate (GTP), and 0.5 ethylene glycol-bis-(β-aminomethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) or 145 CsCl, 0.1 CaCl\(_2\), 2 MgCl\(_2\), 10 HEPES, 4 Na\(_2\)ATP, 0.4 Na\(_2\)GTP, and 0.5 EGTA. For experiments with bis-(o-aminophenoxy)-N,N',N'-tetraacetic acid (BAPTA), the intracellular solution was (in mM) 110 CsCl, 10 HEPES, 2 MgCl\(_2\), 10 BAPTA, 4 Na\(_2\)ATP, and 0.4 Na\(_2\)GTP. The pH was adjusted to 7.3 with CsOH. After a tight seal (>5 GΩ) was formed, the membrane was broken by suction to achieve the whole cell configuration. All recordings were performed in voltage-clamp using an EPC-7 patch-clamp amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany). The holding potential was set at −70 mV, and the slow capacitance compensation and the G-series adjustment has been challenged by the lack of \([Ca^{2+}]_i\) (Linden et al. 1994; Tempia et al. 1992; Vranesic et al. 1991). Such a hypothesis really is involved in the production of the capacitance transient evoked by hyperpolarizing pulses from −70 to −80 mV. The series resistance was compensated whenever its value exceeded 8–10 MΩ except in the experiments with intracellular blockers for which the uncompensated series resistance was <5 MΩ during all the time of whole cell recording. Data were filtered at 3 kHz, digitized at 10 kHz by an A/D converter (ITC 16, Instrutech, Great Neck, NY) and stored on a Macintosh computer (Quadra 650, Apple Computer, Cupertino, CA) using the Pulse Control software kindly provided by Dr. J. D. Herrington and Dr. R. J. Bookman of the University of Miami (Florida). Data were analyzed off-line by the commercial program IgorPro (WaveMetrics, Lake Oswego, OR). Drugs were applied by changing the perfusion line (exchange time in the chamber: 10–20 s), at the following concentrations: bicuculline, 20 μM; 6-cyano-7-nitroquinoxaline-2,3-dione (CNOX), 20 μM; 6-nitro-7-sulfamoylbenzo[\(\text{f}\)]quinoxaline-2,3-dione (NBQX), 10 μM; (RS)-2-amino-5-phosphonopentoic acid (d-AP5), 50 μM; (RS)-a-methyl-4-carboxyphenylglycine (MCPG), 1 mM; and (RS)-1-aminoindan-1,5-dicarboxylic acid (AIDA), 100, 200, or 500 μM. CNOX, NBQX, d-AP5, MCPG, and AIDA were purchased from Tocris Cookson (Langford, UK); guanosine 5'-O-(2-thiodiphosphate) (GDP-β-S) and heparin (low molecular weight: ~3,000, sodium salt) were bought from Sigma Chemical (St. Louis, MO); bicuculline was bought either from Sigma Chemical or from Research Biochemicals International (Natick, MA); U-73122, PKC inhibitory peptide, 19–36 were bought from Calbiochem-Novabiochem (La Jolla, CA).

**Results**

Identification of a slow EPSC evoked by parallel fiber stimulation (mGluR-EPSC)

Purkinje cells were whole cell recorded in the presence of bicuculline (20 μM) in the bath perfusion solution to
block γ-aminobutyric acid-A (GABA_A) receptors, and parallel fibers were stimulated by an extracellular electrode placed in the molecular layer. Stimulation with a single shock evoked an EPSC (AMPA-EPSC; Fig. 1A) that was blocked by an antagonist of AMPA and kainate receptors (NBQX, 10 μM). When, instead of a single shock, a brief train of stimulation pulses was applied to parallel fibers, the response became larger and longer-lasting, in part because of summation of AMPA-EPSCs, but in addition, a second component appeared with a slower time course (Fig. 1A). Whereas the first component was sensitive to NBQX (10 μM; Fig. 1A), the slower component was not affected by this drug, but it was largely inhibited by the mGluR antagonist (RS)-MC PG (1 mM; Fig. 1B).

To isolate the metabotropic component of the parallel fiber-evoked EPSC (mGluR-EPSC), all subsequent recordings were performed with iGluR blockers present in the bath [for AMPA and kainate receptors CNQX (20 μM) or NBQX (10 μM)], for NMDA receptors D-AP5 (50 μM); Fig. 1C]. In these conditions, in the cases where the AMPA-EPSC was largely inhibited but still visible, the mGluR-EPSC was always clearly distinct from the AMPA-EPSC because it had a longer latency and a slower time course and it usually began when the AMPA-EPSC already had finished and the membrane current returned to its basal level (Fig. 1C). The mGluR-EPSC also was present in Purkinje cells in which the block of the AMPA-EPSC by iGluR antagonists was complete. All studies of parallel fiber-evoked mGluR-EPSCs were performed in Purkinje cells in which no previous climbing fiber response was elicited unless otherwise specified.

A detailed analysis was performed on mGluR-EPSCs evoked by four pulses at 100 Hz. In each cell at the beginning of the experiment, when only bicuculline was present in the bath, the stimulation intensity was set at a value that gave an AMPA-EPSC 78–250 pA in amplitude. After the addition of iGluR antagonists, the peak amplitude of the mGluR-EPSC had a mean of 123 ± 88 (SD) pA (n = 71). The latency of the mGluR-EPSC, measured from the beginning of the first stimulus to the beginning of the response, ranged between 0.14 and 0.78 s (mean 0.29 ± 0.13 s, n = 71), the rise time from the beginning to the peak of the response was 0.55 ± 0.41 s (n = 71), and the overall duration was 3.2 ± 1.2 s (n = 71).

**Failure of climbing fiber stimulation to evoke a mGluR-EPSC**

In four Purkinje cells, climbing fiber responses were evoked by a stimulus that was below threshold for activation of parallel fibers (Fig. 2, A and B). In the presence of the cocktail of ionotropic receptors blockers, the climbing fiber was stimulated with trains of ≈20 pulses at 100 Hz. mGluR responses were not evoked by such stimulation in any of these cells (Fig. 2C), indicating that climbing fiber activation of mGluR is either absent or not coupled to the induction of the mGluR-mediated inward current. The same result was obtained for the mGluR-EPSP with intracellular recordings by Batchelor and Garthwaite (1997). To confirm that the lack of response was not due to a nonspecific alteration of tested Purkinje cells, after the assessment of the lack of mGluR-EPSC after climbing fiber activation, the stimulating electrode was moved to the molecular layer and parallel fibers were stimulated. Also Purkinje cells that did not respond to climbing fiber stimulation showed a mGluR-EPSC when parallel fibers were stimulated (Fig. 2D). Thus in Purkinje cells, the coupling of mGluR1 to the mGluR-EPSC is segregated functionally to parallel fiber synapses.
0.999, and 0.973, and all of them were significantly linear (Student’s t-test, $P < 0.05$, $P < 0.001$, $P < 0.001$).

To establish the effect of the frequency of the pulses in the train on the mGluR-EPSC amplitude, the number of pulses was kept constant ($6$ in the example of Fig. 3, C and D) while the frequency was varied. The response began at a threshold of $\sim 10$ Hz, gradually rose until it reached a maximum at $100$ Hz and declined at higher frequencies (Fig. 3, C and D).

**Effects of mGluR antagonists on the mGluR-EPSC**

Application via the bath of the mGluR antagonist (RS)-MCPG ($1$ mM), which is a nonspecific mGluR antagonist active on mGluR subtypes of group I and II (Pin and Duvoisin 1995), greatly inhibited the mGluR-EPSC (to $7.1 \pm 9.1\%$, $n = 7$; Fig. 4A). The group I mGluR antagonist AIDA inhibited the mGluR-EPSC in a dose-dependent manner with an effect $\geq 50\%$ at $200$ $\mu$M ($n = 5$, Fig. 4B), suggesting that the current is mediated by receptors of mGluR1 subtype (see DISCUSSION). While the effect of (RS)-MCPG usually could be washed out completely in $2$–$8$ min, the recovery from the inhibition produced by AIDA was slow and usually not complete (data not shown).

**Effects of blockers of the putative intracellular transduction pathway**

The metabotropic glutamate receptor responsible for the mGluR-EPSC, mGluR1, when expressed in heterologous

![](image_url)

**FIG. 2.** Failure of climbing fiber stimulation to evoke a metabotropic glutamate receptors (mGluR)-EPSC. A: $2$ superimposed traces obtained with the same stimulus intensity that resulted in a full climbing fiber response and a failure. B: climbing fiber stimulation during block of AMPA and kainate receptors by NBQX ($10$ $\mu$M). Stimulus intensity was set at a value higher than the threshold to be sure of activating the climbing fiber. C: climbing fiber stimulation by a train of $12$ pulses at the same intensity as in B shown on a more compressed and amplified time scale. Note the complete absence of any mGluR-EPSC. D: parallel fiber stimulation with $4$ pulses, delivered to the same cell, obtained after moving the stimulation electrode to the molecular layer. Note the presence of a mGluR-EPSC evoked by parallel fiber activation. Calibration shown in the bottom applies to C and D.

In another group of nine Purkinje cells, the stimulation activated both climbing and parallel fibers. In these cells, after the addition of ionotropic glutamate receptors blockers, a mGluR-EPSC could be evoked, indicating that simultaneous climbing fiber activation did not prevent the mGluR response due to parallel fibers.

**Effect of number and frequency of stimulation pulses on mGluR-EPSC**

Parallel fiber stimulation with a single shock was usually not enough to evoke the mGluR-mediated response. The amplitude of the mGluR-EPSC depended on both the number and the frequency of shocks in the train of pulses delivered to parallel fibers (Fig. 3, A and B). The detection capability over the number of pulses above threshold was nearly linear and extended to $>10$ pulses without reaching a plateau; this is at variance with the saturation observed with intracellular recordings (Batchelor and Garthwaite 1997). The linear regression analysis resulted for all cells ($n = 10$) in correlation coefficients $r$ ranging between $0.985$ and $0.999$ that were all statistically significant (Student’s t-test, $P < 0.001$). Because the mGluR-EPSP was found to saturate with $>6$–$10$ pulses (Batchelor and Garthwaite 1997), a linear regression also was performed on the responses to $\simeq 10$ pulses. In three Purkinje cells, the correlation coefficients $r$ were $0.997$, $0.999$, and $0.973$, and all of them were significantly linear (Student’s t-test, $P < 0.05$, $P < 0.001$, $P < 0.001$).

FIG. 3. Effect of number and of frequency of pulses of parallel fiber stimulation on the mGluR-EPSC. A: mGluR-EPSCs evoked in a Purkinje cell by stimulations at $100$ Hz with different durations: $9$ traces are superimposed and were obtained with $3$, $4$, $6$, $8$, $10$, $12$, $14$, $16$, and $18$ pulses. B: plot of peak amplitude of the mGluR-EPSC as a function of the number of pulses in the tetanus. Each symbol represents a Purkinje cell. +, cell shown in A. C: mGluR-EPSCs evoked in a Purkinje cell by stimulations of $6$ pulses with frequencies of $12.5$, $25$, and $100$ Hz. D: mGluR-EPSC peak amplitude vs. pulse frequency plot. Data points represent averages of results from $12$ Purkinje cells, normalized to the response at $100$ Hz. Bars are standard errors of the mean.
systems is coupled to a G protein that activates the membrane enzyme PLC (Houamed et al. 1991; Masu et al. 1991; Pin and Duvoisin 1995). This enzyme catalyzes the breakdown of phosphatidyl inositol diphosphate (PIP2) into inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 is a diffusible second messenger that, acting on intracellular receptor-channels called IP3-receptors (IP3-Rs), mobilizes calcium from intracellular stores, whereas DAG production leads to the activation of PKC.

To assess whether this second-messenger pathway was involved in the mGluR-EPSP, the effects of inhibitors of G proteins, PLC, IP3-Rs, and PKC were tested. In addition, the role of [Ca2+]i, was assessed using a high intracellular concentration of a calcium buffer (BAPTA, 10 mM). To block PLC, we used a compound (U-73122) that is membrane permeable and therefore can be applied via the bath perfusion. The blockers used for the other elements of the transduction pathway and the intracellular Ca2+ buffer are not membrane permeable and were added to the pipette solution at concentrations higher than those required to obtain an effect when applied directly to their target molecules. To allow a fast rate of diffusion from the recording pipette into the cytoplasm, the series resistance was kept <5 MΩ for the whole duration of the recording. The amplitude of the mGluR-EPSP was monitored starting 5 min from the establishment of the whole cell configuration, that is, the time required to obtain an AMPA-EPSP and to block it by the antagonists cocktail. Using the control intracellular solution, without any blocker, the mGluR-EPSP had a slight rundown during the first 30 min of recording (n = 4; Fig. 5A, ◊). This was used as a baseline to test the effects of the blockers.

G PROTEIN. After establishing that the mGluR-EPSP is mediated by mGluR1, we tested whether it requires the activation of a G protein, as expected for the normal function of a metabotropic receptor. A reliable method to test the involvement of a G protein is to use GDP-β-S, a nonhydrolyzable analogue of GDP that binds to all G proteins, locking them in an inactive state. In Purkinje cells recorded with a pipette solution containing 1 mM GDP-β-S, the amplitude of the mGluR-EPSP started to drop after ~10 min of whole cell recording (n = 4; Fig. 5A, □). At 10 min the difference between control and GDP-β-S was already statistically significant (Student’s t-test, P < 0.001) and remained significant after 15 and 20 min of recording (Student’s t-test, respectively, P < 0.002 and P < 0.001). The time course of this drop is likely to reflect the time for diffusion of GDP-β-S from the pipette to the sites where mGluR1-coupled G proteins are located. This result indicates that a G protein is required to mediate the mGluR-EPSP response.

PLC. The membrane-permeable PLC inhibitor U-73122 (2 µM) was applied via the bath perfusion for 1–3 min to 11 Purkinje cells. Instead of the block of the mGluR-EPSP, which was expected, the amplitude of the mGluR-EPSP was 99.6 ± 6.5% (mean ± SE, n = 11) of the control. The mean amplitudes of the mGluR-EPSP before and during application of U-73122 are shown in Fig. 5B. There is no significant change in amplitude (paired t-test, P > 0.90). This result indicates that PLC is not necessary for the mGluR-EPSP.

IP3 RECEPTOR. A high concentration of heparin (low molecular weight, 7 mg/ml) was included in the pipette solution to block IP3-Rs. The recording requirements for experiments with intracellular blockers were obtained in five Purkinje cells, for which the time course of the mGluR-EPSP is shown in Fig. 5A (△). In the case of GDP-β-S, most of the effect was observed in the first 10 min of recording, but heparin has a higher molecular weight (~3,000). Therefore, a concentration much larger than required was chosen, and the amplitude of the mGluR-EPSP was followed for 30 min to maximize the possibility of seeing any effect. Only a small tendency to decrease, not significantly different from the control, was present (Fig. 5A, △), suggesting that, similarly to PLC, IP3-Rs are not involved in the mGluR-EPSP.

PKC. To assess whether the PKC pathway is involved in the mGluR-EPSP, the PKC inhibitory peptide 19–36 (10 µM), which is a specific pseudosubstrate amino acid sequence of the regulatory domain of PKC (House and Kemp 1987), was added to the pipette solution. With this solution, five Purkinje cells were recorded for 30 min under the same experimental conditions as with the other intracellular blockers used. The time course was not significantly different from controls (Fig. 5A, ◊), and therefore PKC also can be considered not to be involved in the generation of the mGluR-EPSP.

[Ca2+]i. To test the role of a possible transient increase in [Ca2+]i, occurring during the mGluR-EPSP, five Purkinje cells were recorded, under the same conditions as with the intracellular blockers, with an intracellular solution containing 10 mM BAPTA (Fig. 5A, ○). The time course of the mGluR-EPSP amplitude was intermediate between that of the control and that obtained with GDP-β-S. From 10 to 25 min of whole cell recording, the difference relative to the control was statistically significant (Student’s t-test, P < 0.05).

DISCUSSION

In the present paper, we describe for the first time the recording of an inward current, evoked by parallel fiber stim-
ulation, which is mediated by a mGluR in cerebellar Purkinje cells under voltage-clamp conditions. This current (mGluR-EPSC) is evoked by stimulation similar to that used to obtain a depolarizing response in L-shaped cerebellar slices (Batchelor and Garthwaite 1993) and an intracellularly recorded EPSP (mGluR-EPSP) (Batchelor and Garthwaite 1997; Batchelor et al. 1994, 1996, 1997). Therefore it can be concluded that the current that we describe is the main determinant of this recently discovered mGluR-EPSP. The kinetic parameters of the mGluR-EPSP described in this paper are similar to those of the mGluR-EPSP (Batchelor and Garthwaite 1997). The latency and the time to reach the peak of the response are very similar, whereas the overall duration of the inward current was on average longer than the EPSP (3.2 vs. 1.5 s). A possible explanation of the shorter duration of the EPSP could be the involvement of active conductances that might truncate the response.

The lack of response to climbing fiber stimulation confirms the observation obtained with intracellular recording (Batchelor and Garthwaite 1997). Such a finding is surprising, because, by electronmicroscopic immunohistochemistry, it has been shown that mGluR1 receptors are present perisynaptically at both parallel and climbing fiber synapses (Nusser et al. 1994). A possible explanation is that mGluR1 close to climbing fiber synapses has a different coupling to second messengers than mGluR1 close to parallel fiber synapses.

With increasing number of pulses in the stimulation train given to parallel fibers, the depolarizing response extracellularly recorded in L-shaped cerebellar slices saturates with six pulses (Batchelor and Garthwaite 1993; Batchelor et al. 1994). The mGluR-EPSP recorded with an intracellular electrode also saturates with 6–10 pulses (Batchelor and Garthwaite 1997). In our experimental conditions, such saturation is not present and, even with >10 pulses, the mGluR-EPSC continues to grow in a fairly linear fashion (Fig. 3B). A response recorded under voltage clamp better reflects the activation of mGluR in the Purkinje cell. During parallel fiber stimulation with a train of pulses at the same frequency as used in our experiments, the summation of Ca$^{2+}$ in parallel fiber axon terminals is nearly linear (Mintz et al. 1995; Regehr and Atluri 1995). However, it has been shown that the release of glutamate by parallel fibers is a supralinear function of Ca$^{2+}$ influx (Mintz et al. 1995). A saturating response is the opposite of what would be expected to result from a supralinear glutamate release, although it would be possible that all receptors had been recruited by the first 6–10 pulses of stimulation or that the release of high amounts of neurotransmitter was enough to produce a relevant receptor desensitization. The absence of saturation of the mGluR-EPSP with >10 pulses indicates that, during the development of the response, receptor desensitization is not prominent and that even with 10 pulses there are still receptors available to give a further contribution. The saturation observed by Batchelor and Garthwaite (1993, 1997) and Batchelor et al. (1994) therefore should be attributed to the type of stimulation that they used or to a contribution of active conductances to the responses recorded extracellularly or to the potentials recorded intracellularly. Although the necessity for more than one pulse could be attributed to the requirement for glutamate to reach the nearest receptors, located at the periphery of the synapse (Nusser et al. 1994), before being taken up by the transporters, the absence of saturation could be explained by the presence of receptors at extrasynaptic locations, even distant from the synapse (Nusser et al. 1994), which are recruited only when large amounts of glutamate are released by parallel fibers.

A half-maximal inhibition of the mGluR-EPSP by 200 µM AIDA is in agreement with the IC$_{50}$ of 62 µM found when this drug was tested on mGluR1 (Moroni et al. 1997). The slow and incomplete washout of the effect of AIDA also was observed by Batchelor et al. (1997) and might be due to a difficulty of this drug in diffusing through the tissue surrounding Purkinje cell dendrites. The effect of AIDA on
the other receptor of group I (mGluR5) is much weaker (Moroni et al. 1997), and on receptors coupled to adenylyl cyclase inhibition, it has only a modest agonist activity at large concentrations. This finding indicates that the mGluR-EPSC is mediated by mGluR1 and not by the only other mGluR known to be expressed by Purkinje cells, mGluR7, which is coupled to adenylyl cyclase inhibition (Kinzie et al. 1995; Ohishi et al. 1995). This identification of the receptor responsible for the mGluR-EPSC as mGluR1 is in agreement with its abundant expression in adult Purkinje cells (Masu et al. 1991). A pharmacological study conducted on the mGluR-EPSP led to the same conclusion (Batchelor et al. 1997). The mGluR-EPSC was present in rats of all ages used for these experiments, including the earliest age of 15 days. However, the mGluR-mediated depolarizing response recorded with an extracellular technique was not found in slices from 12- to 17-day-old rats but was present in rats >4 wk old (Batchelor and Garthwaite 1993). Actually, in rat Purkinje cells, at postnatal day 3, mGluR1 is just detectable, and its expression gradually increases during postnatal development until reaching a high level 21 days from birth that then is maintained in adulthood (Casabona et al. 1997; Catania et al. 1994; Condorelli et al. 1992; Shigemoto et al. 1992; Van den Pol et al. 1994). Thus the late appearance in development of the mGluR-mediated response, at the beginning of the third postnatal week (present study) or slightly later (Batchelor and Garthwaite 1993), is in agreement with the developmental expression of mGluR1.

Our results on the second messengers mediating the mGluR-EPSC confirm the requirement of a G-protein but show that the transduction pathway that is followed is not the same as that described in heterologous expression systems (Houamed et al. 1991; Masu et al. 1991). The possibility that the lack of effect of some blockers could be due to problems in reaching the sites where they have to act was accurately considered. The PLC blocker U-73122 was used at the highest concentration that did not elicit significant nonspecific effects (2 μM). In fact, it has been shown that this compound induces release of Ca2+ from intracellular stores primarily through inhibition of the internal Ca2+ pump (De Moel et al. 1995). The nonspecific effect observed in our experiments with larger concentrations consisted of a potentiation of the mGluR-EPSC that developed within 1 min from the beginning of the application of U-73122 (n = 3; data not shown). This is in agreement with the finding that an increase in [Ca2+]i, potentiates the mGluR-mediated response (Batchelor and Garthwaite 1997). More importantly, the presence of a clear effect already after 1 min indicates that this lapse of time was sufficient for this blocker to cross the plasma membrane, reach the interior of the cell, and act on Ca2+ stores. Therefore our results exclude the possibility that there was insufficient time for U-73122 to reach the membrane and block PLC. The possibility that a nonspecific potentiation could have counteracted and masked a specific inhibition was excluded by the fact that a lower concentration (0.5 μM) also did not cause any change in the mGluR-EPSC (n = 2; data not shown). Regarding heparin, the slower diffusion along the dendrites was more than compensated by the very high concentration used and by the threefold longer time during which the responses were monitored. However, because heparin is not a strong antagonist of IP3-Rs and in addition has other side effects, the role of IP3-Rs in the mGluR-EPSC needs further consideration. Furthermore, IP3 could have effects, independent from those due to the release of Ca2+ from internal stores and not affected by heparin, that could play a role in the modulation of the mGluR-EPSC. The PKC inhibitory peptide was effective in blocking LTD in Purkinje cells recorded in whole cell configuration when it was applied for 5–20 min before the induction (Linden and Connor 1991). The same concentration (10 μM) did not result in any significant change in the mGluR-EPSC.

The effect of dialyzing the cell with a high concentration of BAPTA, clamping the [Ca2+]i to negligible levels, resulted in a decline of the mGluR-EPSC with a behavior intermediate between the control and the block by GDP-β-S. This result suggests that with BAPTA, the mGluR-EPSC is set to a completely unpotentiated state, but it is not blocked. On one hand, this indicates that a rise in [Ca2+]i, is not a necessary element of the pathway responsible for the mGluR-EPSC; on the other hand, it is in agreement with a role of [Ca2+]i, in enhancing the gain of the response (Batchelor and Garthwaite 1997; Tempia et al. 1997). Furthermore, the decrease of the mGluR-EPSC amplitude produced by clamping the [Ca2+]i, at a concentration very close to zero suggests that normal resting [Ca2+]i, levels already exert a certain degree of potentiation of the mGluR-EPSC. This explains previous observations in which a Ca2+ buffer introduced into Purkinje cells by intracellular injection or by loading a membrane permeable form of BAPTA inhibited the responses to applications of a mGluR agonist (Linden et al. 1994; Staub et al. 1992).

Thus our results rule out the involvement of the main elements of the putative transduction pathway, including PLC, [Ca2+]i, and PKC in the production of the mGluR1-mediated postsynaptic current. This result is at variance with the recent finding that PLC is necessary for the responses to mGluR agonists applied to Purkinje cells (Netzeband et al. 1997). It is possible that mGluR agonist application involves mGluRs located far from parallel fiber synapses and coupled with the PLC pathway or that mGluR agonists also activate some still unknown receptors coupled with PLC activation. In agreement with our findings, recent data indicate that the IP3 response to mGluR agonists in the brain is not mediated by mGluR1 but by mGluR5, suggesting that in neurons, at variance with heterologous expression systems, mGluR1 is not coupled to PLC but to other effectors that have not yet been identified (Casabona et al. 1997). However, the lack of involvement, in our results, of the PLC pathway for the mGluR-EPSC does not exclude that in Purkinje cells mGluR1 can mediate other types of responses through the PLC pathway.

Alternative transduction pathways activated by mGluR1 have been shown in a variety of systems. Modulation of potassium channels by group I mGluRs in a PKC/PKA-independent mechanism was shown in hippocampal neurons and in neurons of the nucleus of the tractus solitarius (reviewed by Pin and Duvoisin 1995). Cyclic guanosine monophosphate was shown to mediate group I mGluRs modulation of GABA_A and AMPA receptors (reviewed by Pin and Duvoisin 1995). In a recent study using a heterologous expression system, mGluR1 was shown to modulate N-type
and P/Q-type Ca\(^{2+}\) channels by coupling to G\(_i/G_o\)-like proteins through both voltage-dependent and Ca\(^{2+}\)-dependent pathways (McCool et al. 1998).

For the mGluR-EPSC, a transduction pathway different from that involving PLC explains several previous observations, such as the lack of [Ca\(^{2+}\)]\(_i\) increase associated with the mGluR-EPSP (Batchelor et al. 1996) or the absence of response after the [Ca\(^{2+}\)]\(_i\) increase evoked by climbing fiber stimulation (Batchelor and Garthwaite 1997). Moreover, the fact that a release of Ca\(^{2+}\) from intracellular stores is not directly responsible for the generation of the mGluR-EPSC, together with a negligible Ca\(^{2+}\) permeability of AMPA receptors of Purkinje cells (Tempia et al. 1996a) confines the role of producing or at least initiating any increase in [Ca\(^{2+}\)]\(_i\), to voltage-gated Ca\(^{2+}\) channels. On the other hand, after a first rise in [Ca\(^{2+}\)]\(_i\), IP\(_3\)-sensitive Ca\(^{2+}\) stores could participate in the amplification of the Ca\(^{2+}\) signal by means of the Ca\(^{2+}\) sensitivity of IP\(_3\) receptors and with a possible contribution of ryanodine receptors (Berridge 1996).

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