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# Pharmacological and Immunocytochemical Characterization of Metabotropic Glutamate Receptors in Cultured Purkinje Cells

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**Metabotropic glutamate receptor (mGluR) is highly expressed in cerebellar Purkinje cells. The purpose of this study was pharmacological and immunocytochemical characterization of the mGluR in single cerebellar neurons, especially Purkinje cells. $Ca^{2+}$ imaging with fura-2 in cultured cerebellar neurons, identified immunocytochemically, was used to record the direct effects of drugs in stable conditions. In addition, the expression of mGluR was examined, and expression of the intracellular receptor for inositol trisphosphate ($IP_3$) produced by mGluR activation was studied immunocytochemically with specific antibodies. Purkinje neurons and some other neurons showed $Ca^{2+}$-mobilizing responses to mGluR agonists. These responses were mediated by mGluR because they were not blocked by ionotropic GluR antagonists, were independent of the caffeine-sensitive $Ca^{2+}$ pool, and were blocked by inhibitors of $IP_3$-induced $Ca^{2+}$ release. This is the first pharmacological characterization of mGluR at single Purkinje cells. The results differed as follows from those in earlier studies in which phosphoinositide turnover of the entire population of cerebellar cells was monitored: (1) the mGluR responses were not blocked by pertussis toxin or D,L-2-amino-3-phosphonopropionic acid; (2) glutamate was a potent agonist, whereas L-aspartate was ineffective; and (3) the dose–response relationship showed an all-or-none tendency. The metabotropic response of Purkinje cells changed markedly during development, with a sharp peak after day 4 of culture, whereas mGluR and $IP_3$ receptor proteins increased steadily during maturation. This apparent desensitization of mGluR was not blocked by inhibitors of protein kinase C (PKC) or ADP-ribosyltransferase. The metabotropic responses were mainly localized to the center of the somata of Purkinje cells even on day 4, whereas both receptor proteins were expressed throughout the cell. These results suggest that the function of mGluR is spatially and developmentally controlled by a posttranslational mechanism involving a mechanism other than phosphorylation by PKC or ADP-ribosylation.**

The excitatory amino acid (EAA) glutamate plays a crucial role in synaptic plasticity and the pathogenesis of brain damage associated with anoxia, hypoglycemia, epilepsy, and some neurodegenerative diseases (for review, see Monaghan et al., 1989; Meldrum and Garthwaite, 1990). There are two main classes of glutamate receptors: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs) (Sugiyama et al., 1989). The iGluRs are further classified according to their preferential agonists into NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolone propionate (AMPA), kainate, and L-amino-4-phosphonobutanoate receptors. Glutamate stimulates $Ca^{2+}$ entry into neurons through both voltage-sensitive $Ca^{2+}$ channels and iGluR channels (mainly NMDA subtype) (Kudo and Ogura, 1986; MacDermott et al., 1986; Ozawa et al., 1988). Increase in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) through these pathways is considered to be an essential step in the mechanisms of neuronal plasticity and neurotoxicity. The mGluR is linked to GTP-binding proteins (G-proteins), which regulate phospholipase C (PLC) and subsequent production of the intracellular messengers inositol trisphosphate ($IP_3$) and diacylglycerol (see Schoepp et al., 1990a, for a review). The major function of $IP_3$ is thought to be the mobilization of $Ca^{2+}$ from intracellular stores (Berridge, 1987). Recently, Masu et al. (1991) cloned the cDNA for mGluR and demonstrated prominent expression of mRNA for the receptor in the hippocampal and cerebellar neurons, where two major types of synaptic plasticity, long-term potentiation (LTP) (Nicoll et al., 1988) and long-term depression (LTD) (Ito, 1989), respectively, can be evoked. Together with recent reports (Ito et al., 1988; Goh and Pennefather, 1989; Ito and Karachot, 1990; Linden et al., 1991), their result strongly supports the view that mGluRs also play an important role in synaptic plasticity by mobilizing $Ca^{2+}$ from internal stores. The functional characterization of mGluRs in these neurons are therefore of much importance.

The mGluRs have been characterized mainly in brain slices, synaptoneurosomes, and cultured brain cells with phosphoinositide (PI) breakdown as a marker. In these studies, however, the responses of populations of cells were monitored and may have consisted of heterogeneous responses of several types of neurons and astrocytes and of presynaptic mGluRs (Adamson et al., 1990). A more serious problem is that $Ca^{2+}$ entry through voltage-dependent $Ca^{2+}$ channels and NMDA channels can ac-

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tivate PLC, leading to PI breakdown that is not caused by the activation of the mGluRs. Classical electrophysiological techniques were applied in single neurons (Stratton et al., 1990) as well as in *Xenopus* oocytes expressing mGluRs (Sugiyama et al., 1989), but $Ca^{2+}$ mobilization could be detected only indirectly, for example, by a $Ca^{2+}$-activated $Cl^-$ current. Recently, Llano et al. (1991) demonstrated $Ca^{2+}$ mobilization by quisqualate by direct $Ca^{2+}$ measurement with fura-2 in Purkinje cells in cerebellar slices, but pharmacological data are still lacking. This may be due to the difficulty of stable recording in slice preparations.

An alternative approach is the use of cultured neurons. There are, however, two major technical problems in this approach: how to identify specific cell types and how to establish culture conditions that facilitate normal cell differentiation. The former problem can be overcome by immunocytochemical identification of cell types (Gruol and Crimi, 1988; Yool et al., 1988; Hockberger et al., 1989; Yuzaki et al., 1990) or by isolated culture of a specific cell type (Brorson et al., 1991). With respect to the second problem, earlier works have shown that several receptor channels develop in cultured Purkinje cells in the same way as *in vivo* (Gruol and Crimi, 1988; Yool et al., 1988; Hockberger et al., 1989), indicating that the expression of postsynaptic receptors in Purkinje cells is mainly programmed intrinsically. Nevertheless, the results obtained in cultures should be carefully interpreted in comparison with those *in vivo*.

The purpose of this study was pharmacological and immunocytochemical characterization of the mGluR in single cerebellar neurons, especially Purkinje cells. We used $Ca^{2+}$ imaging with fura-2 in cultured cerebellar neurons to record the direct effect of drugs in more stable conditions. This preparation also permits the investigation of change of functional mGluR during maturation. Cells were identified immunocytochemically. Normal differentiation of neurons in culture was confirmed by immunocytological staining of $IP_3$ receptor ($IP_3R$) and mGluR proteins as well as by comparing the results with those *in vivo*.

## Materials and Methods

*Cell culture.* Cerebellar neurons were prepared from ICR mice as described previously (Yuzaki et al., 1990) with minor modifications to obtain a Purkinje cell–rich culture. Briefly, cerebella from embryos on gestational day 18–19 were treated with 0.1% trypsin (Difco) and 0.05% DNase I (Sigma) in $Ca^{2+}$/$Mg^{2+}$–free Hanks' balanced salt solution (HBSS) (Sigma) for 5 min at 25°C. The cells were washed with culture medium containing 1 μg/ml of the trypsin inhibitor aprotinin (Sigma), dissociated by repeated passage through a fine-tipped pipette in $Ca^{2+}$-free HBSS containing 0.05% DNase I and 12 mM $MgSO_4$, and then rinsed with culture medium. Dispersed cells were plated at a density of 20–25 × $10^4$ cells/$cm^2$ onto poly-L-lysine (Sigma)–coated glass coverslips (Matsunami; 0.15 mm thick) in serum-free defined medium (Fischer, 1982): Eagle's medium supplemented with 1 mg/ml bovine serum albumin (BSA), 10 μg/ml insulin, 0.1 nM L-thyroxine, 0.1 mg/ml transferrin, 1 μg/ml aprotinin (all from Sigma), 30 nM selenium (Merck), 0.1 mg/ml streptomycin (Meiji), and 100 U/ml penicillin (Banyu). The cultures were maintained in a humidified atmosphere of 5% $CO_2$ in air at 37°C.

In some experiments, pertussis toxin (PTX) (Funakoshi), γ-aminobutyric acid (GABA), sphingosine, nicotinamide, or polymyxin B (all from Sigma) was added to the culture medium for an appropriate period. PTX and nicotinamide were directly dissolved in culture medium. GABA and polymyxin B were dissolved in water as 1000× solution. Sphingosine was dissolved in hot ethanol with a drop of $H_2SO_4$ as 1000× solution.

*Microfluorometry.* Fura-2 loading and video-assisted $Ca^{2+}$ microfluorometry were performed as described previously (Yuzaki et al., 1989). Briefly, cells cultured on coverslips were exposed to recording solution (137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, buffered at pH 7.3 with 20 mM $Na^+$-HEPES) containing 2.5 μM fura-2 acetoxymethyl ester (Dojin) for 60 min at 37°C and then washed twice with fresh recording medium.

Temporal changes in the fluorescence image at a wavelength of 340 nm were viewed through a silicon-intensifier target tube (SIT) camera (Hamamatsu Photonics, C2400-8) connected to the camera portion of an inverted microscope (Olympus, IMT2), and recorded on magnetic tape. Brief images (0.3 sec for each) excited at 360 nm were also taken at regular intervals (usually of 3–4 min) to estimate the bleaching of the dye. After immunocytochemical identification of the cell type, the ratio of the fluorescence intensity at 340 nm to that at 360 nm was calculated from video tapes with a computer (NEC, PC-9801 RA21) equipped with a video frame memory (Hamamatsu Photonics, DVS-3000). Using a computer program (Mitsubishi-kasei, FC-300) improved by Dr. T. Inoue in our laboratory, we processed from 28 windows simultaneously, and compensated the bleaching of the dye and offset fluorescence level. Each window consisted of 4 × 4 pixels and corresponded to the somata of the neurons. This method has the advantage of requiring less processing time than the usual method used in many image processors in which entire video frames were calculated pixel by pixel. For spatial analysis at the single-cell level, a digital image processor and an attached computer program (Hamamatsu Photonics, ARGUS-100) were used.

The concentration of $Ca^{2+}$ was calibrated by comparison with the fluorescence ratio of fura-2 free acid in Ca-EGTA-PIPES (Sigma) buffer excited at the two wavelengths as described previously (Yuzaki et al., 1989). Recordings were made at 24–26°C.

*Drug application.* Cells on coverslips were perfused continuously with recording solution at 3 ml/min in a chamber of 0.6 ml volume. Drugs were added to this solution. The capillary tube (1 mm in diameter) for the inlet of solution was placed 1 mm above the cells examined. This system enabled rapid and homogeneous application of drugs to these cells and prevented significant accumulation of endogenous glutamate in the bath.

For high-$K^+$ solutions, 50 mM NaCl was replaced by 50 mM KCl. $Ca^{2+}$-free solution was obtained by adding 5 μM EGTA to nominally $Ca^{2+}$-free medium. $Mg^{2+}$ was omitted from the medium when NMDA was tested, to avoid the blockade of NMDA receptors by this ion. NMDA, kainate, quisqualate, ibotenate, L-glutamate, D,L-2-amino-5-phosphonovalerate (D,L-APV), D,L-2-amino-3-phosphonopropionic acid (D,L-AP3), 3,4,5-trimethoxybenzoic acid-8-(diethylamino)octyl ester (TMB-8) (from Sigma), *cis*-1-aminocyclopentane-1,3-dicarboxylic acid (*trans*-ACPD), AMPA, and L-AP3 (from Tocris Neuramin) were dissolved in water as 500–1000× stock solutions (the pH was adjusted when necessary) and frozen at −40°C until use. 6-Nitro-7-cyanoquinoxaline-2,3-dione (CNQX) (Tocris Neuramin) was dissolved in 50% dimethylsulfoxide (DMSO) as 1000× stock solution and frozen. The presence of 0.05% DMSO had no effect on $Ca^{2+}$ imaging in a control experiment. *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W7) and *N*-(6-aminohexyl)-1-naphthalene sulfonamide (W5) (both from Seikagaku-kogyo) were dissolved in water as 100× stock solutions (pH 4) and stored at 4°C. Caffeine was added as freshly prepared solution. The pH of the final solutions was carefully adjusted because $Ca^{2+}$ measurement with fura-2 was very sensitive to extracellular pH. Tetrodotoxin (Sankyo) at 1 μM was always added to the medium to block the synaptic activity (Yuzaki et al., 1990).

Excess exposure to $Ca^{2+}$-free medium or excess chelating of $Ca^{2+}$ by EGTA resulted in smaller responses, suggesting lability of the storage sites, as has been observed in several other cells (Murphy and Miller, 1988; Ogura et al., 1990). We therefore adopted a protocol in which cells were stimulated by high $K^+$ for 20 sec, returned to normal medium for 10 sec, and perfused with $Ca^{2+}$-free solution for 90 sec before activation of the $Ca^{2+}$-mobilizing receptor to reset the calcium pool at the steady state. The removal of external $Ca^{2+}$ by washing with $Ca^{2+}$-free medium for 90 sec was considered to be sufficient, because after this treatment high-$K^+$ solution did not increase the $[Ca^{2+}]_i$ (see Fig. 2*B*). Using this protocol, we reduced the apparent desensitization of the receptor, and the response to a second application of quisqualate was within 80% of the first response.

*Immunocytochemistry.* To identify Purkinje cells and quantitate $IP_3Rs$, cells were fixed after microfluorometry with 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min, permeabilized with 0.01% Triton X-100 in phosphate-buffered saline (PBS) for 10 min, and blocked with 1% nonfat milk in PBS for 60 min. Cells were incubated with rat monoclonal antibody 18A10 (1 μg/ml) against the $IP_3$ receptor (Maeda et al., 1990) for 60 min and stained by the avidin-biotin complex method with diaminobenzidine as the final substrate for peroxidase (Vectastain). They were viewed through an SIT camera, compared with fluorescence

images recorded on magnetic tapes, and then positively stained cells were marked. In some experiments, cells were further incubated with anti-Spot35/Calbindin-$D_{28k}$ antibody at 1:1000 dilution (a gift from Dr. R. Kuwano, Niigata University), with anti–microtubule-associated protein 2 (MAP2) antibody at 1:500 dilution, or with anti–glial fibrillary acidic protein (GFAP) antibody at 1:1500 dilution (both from Dr. M. Niinobe, Osaka University) for identification of Purkinje cells, neurons, and astrocytes, respectively, and stained with nitroblue tetrazolium chloride as a substrate for avidin-biotin-glucose oxidase (Vectastain). The cells that reacted with anti-Spot35/Calbindin-$D_{28k}$ antibody coincided with those that reacted with 18A10.

For double-immunofluorescent staining of mGluR and $IP_3R$, cells were fixed and permeabilized as described above and blocked with 3% normal goat serum and 0.5% nonfat milk in PBS for 1 hr. They were incubated with blocking solution containing anti-mGluR rabbit antibody (a gift of Dr. Y. Ryo, Osaka University) (10 μg/ml) and 18A10 (5 μg/ml) for 1 hr, and with blocking solution containing fluorescein isothiocyanate (FITC)-labeled anti-rabbit goat IgG and tetramethylrhodamine isothiocyanate (TRITC)-labeled anti-rat goat IgG (both from Kirkegaard & Perry Lab; used at 5 μg/ml) for another 1 hr. Coverslips were mounted on slides in PermaFluro (Immunon) and viewed in a Zeiss Axioplan fluorescent microscope with appropriate filters. The anti-mGluR antibody was raised against synthesized peptide for the Purkinje cell–specific mGluR1 sequence (Masu et al., 1991; Tanabe et al., 1992), and its specificity was ascertained by Western blotting and immunohistochemistry using brain slices (Y. Ryo, A. Miyawaki, T. Furuichi, and K. Mikoshiba, unpublished observations). We also confirmed the specific staining of both antibodies by three control experiments, one in which rabbit IgG (10 μg/ml) was substituted for anti-mGluR antibody, one in which rat IgG (5 μg/ml) was substituted for 18A10, and one in which both rabbit IgG and rat IgG were used.

## Results

### *Culture characteristics*

Culture of cerebellum from embryonic day 18–19 plated at relatively high density (20–25 × $10^4$ cells/$cm^2$) in serum-free medium enabled us to maintain Purkinje cells for 5–6 weeks as well as allowing the functional and morphological development of Purkinje cells. As we reported previously (Yuzaki et al., 1990), neurons begin to show spontaneous synchronous oscillations of intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) from about 2 weeks in such cultures, indicating that they form an active neuronal network *in vitro*. The developmental profile of dendritic arborization in Purkinje cells in culture (Fig. 1) reflected that *in vivo* (Maeda et al., 1989; Nakanishi et al., 1991) to a considerable degree.

Immunological staining with anti-$IP_3R$, anti-GFAP, and anti-MAP2 antibodies revealed that our culture consisted of Purkinje cells (3–4%), astrocytes (1–2%), and small neuronal cells that are most possibly granule cells (93–96%). These ratios remained constant during culture.

### *EAA-induced $[Ca^{2+}]_i$ increase*

Cells loaded with fura-2 responded to applications of glutamate receptor agonists by increases in the fluorescence intensity ratio corresponding to elevations of the $[Ca^{2+}]_i$. The pattern of response was characteristic of the type of cell (Fig. 2*A*). As we reported previously (Yuzaki et al., 1990), Purkinje cells typically showed large responses to quisqualate, moderate responses to kainate, and small responses to NMDA (Fig. 2*A*, left trace), whereas most non-Purkinje cells showed responses of similar size to all the ionotropic EAAs (Fig. 2*A*, middle trace; we refer to this type of response as the "typical type"). In addition, we found some non-Purkinje cells responding only to quisqualate (right trace; 8.7%, 67 of 770; we refer to this response as the "Q type"). The latter cells included those showing little change in $[Ca^{2+}]_i$ even on high-$K^+$ stimulation (40.3%, 27 of 67; "KCl-insensitive Q type"). The non-Purkinje cells that showed the Q-type response including the KCl-insensitive type were probably cerebellar interneurons because they gave a negative reaction for GFAP and a positive reaction for MAP2 and were larger than typical granule cells, but further immunocytochemical studies are needed for their exact identification.

### *Liberation of $Ca^{2+}$ from intracellular stores by quisqualate*

As shown in Figure 2*B*, in $Ca^{2+}$-free medium, quisqualate increased $[Ca^{2+}]_i$ in Purkinje cells, but NMDA, kainate, and AMPA did not. The response to quisqualate in $Ca^{2+}$-free solution was not blocked by the iGluR antagonists CNQX and D,L-APV (Fig. 2*C*), which completely blocked the responses to kainate and NMDA (not shown). Thus, the calcium-mobilizing response by quisqualate was distinct from the response mediated by iGluRs and most possibly mediated by mGluR.

Similar responses to quisqualate were obtained in several types of non-Purkinje cells. The percentage of cells showing a $Ca^{2+}$-mobilizing response to quisqualate were 20.0% (154 of 703) for typical non-Purkinje cells, 57.5% (23 of 40) for Q-type non-Purkinje cells, and 77.8% (21 of 27) for KCl-insensitive Q-type cells, and these percentages remained fairly constant during culture. Cells giving a positive reaction for GFAP showed no $Ca^{2+}$-mobilizing response to quisqualate (0 of 22). The percentage of responsive Purkinje cells varied greatly during culture, as will be described below.

### *Identification of intracellular $Ca^{2+}$ stores*

Some neurons possess an intracellular $Ca^{2+}$ pool that is not triggered by $IP_3$ but by a $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) mechanism (Kuba and Nishi, 1976; Murphy and Miller, 1989; Brorson et al., 1991). Caffeine decreases the threshold $[Ca^{2+}]_i$ for this CICR (Endo, 1975). Caffeine (10 mM) caused $Ca^{2+}$ mobilization in $Ca^{2+}$-free medium in 25 of 34 Purkinje cells and in 16 of 114 non-Purkinje cells on day 4 of culture. The responses were, however, so labile under our experimental conditions that the cells often showed little response to a second treatment with caffeine even after intracellular stores had been reset by KCl exposure. After caffeine-evoked $Ca^{2+}$ mobilization had diminished, the quisqualate-induced $Ca^{2+}$ release was similar to that before caffeine treatment. In rare cases, some cells responded to the repeated exposure to caffeine. In such cells, caffeine caused $Ca^{2+}$ mobilization after quisqualate-induced $Ca^{2+}$ rise had diminished (Fig. 2*E*, left trace) or quisqualate mobilized $Ca^{2+}$ after CICR had reduced (right trace). These results indicate that quisqualate induced $Ca^{2+}$ from an intracellular pool distinct from the CICR-sensitive pool and that the $Ca^{2+}$-mobilizing response observed was mediated by mGluRs coupled with PI turnover.

### *Antagonists of the $Ca^{2+}$-mobilizing response*

To determine whether the receptor was coupled to PTX-sensitive G-protein, we treated the cells with either 1 or 10 μg/ml PTX for 20–22 hr and then examined their response to quisqualate, glutamate, ibotenate, and *trans*-ACPD. The dose–response curves obtained were not significantly different from those for control cells (not shown). Similar results were obtained with Purkinje cells and several types of non- Purkinje cells.

D,L-AP3 is reported as a specific antagonist for an mGluR (see Schoepp et al., 1990a, for review). As shown in Figure 2*D*, D,L-AP3 (1 mM) did not block $Ca^{2+}$ mobilization in response

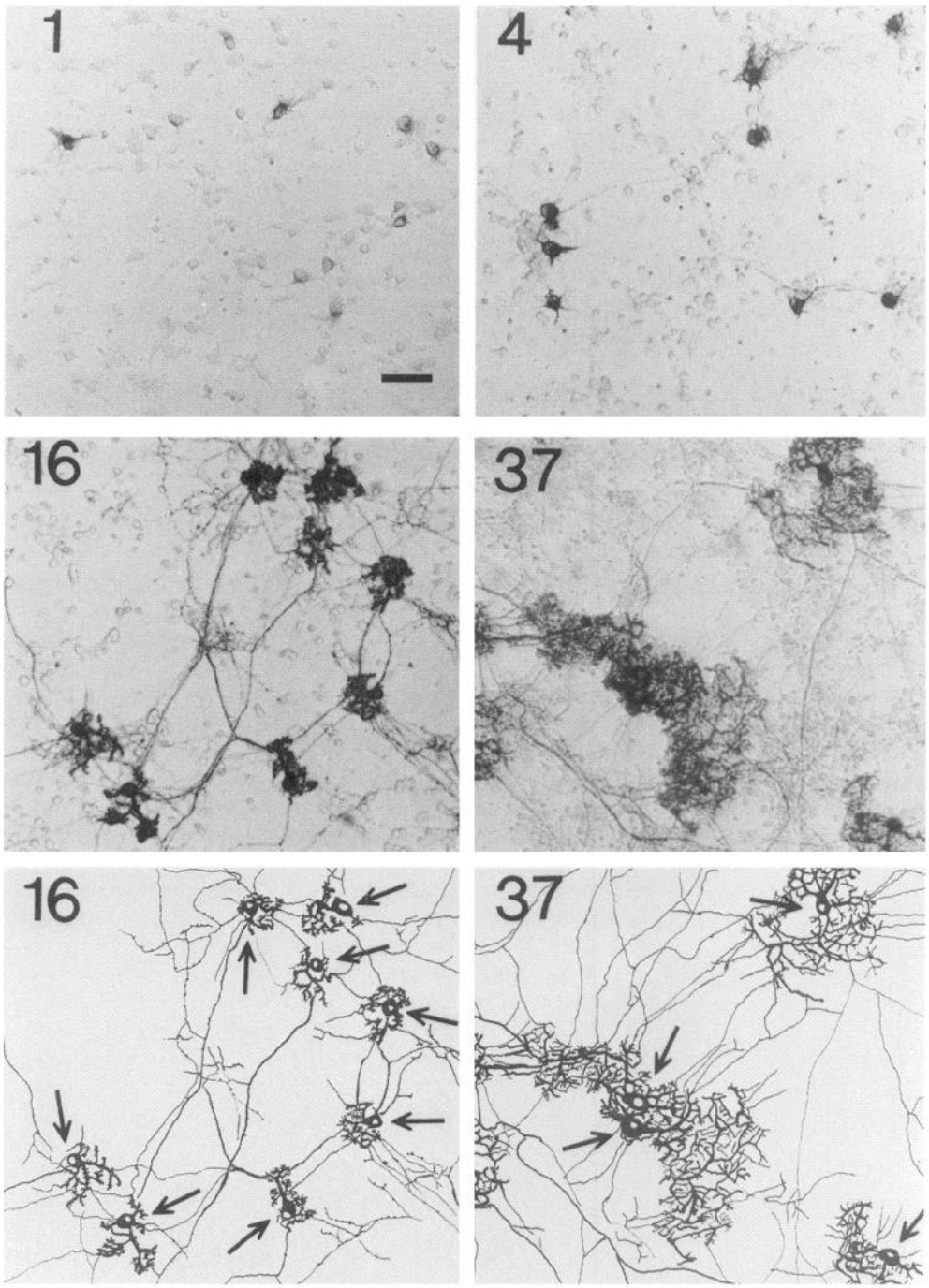



*Figure 1.* Development of Purkinje cells in culture. Cells on the indicated days in culture were stained by the avidin-biotin-peroxidase method with monoclonal antibody 18A10. The *bottom drawings* are traced from the photos on the indicated days to show the dendritic arborization of single Purkinje cells more clearly. *Arrows* indicate the soma of the Purkinje cells. Scale bar, 40 μm.

to quisqualate (1 μM). To confirm this insensitivity to AP3, we conducted a series of studies on the effect of 2 mM L-AP3, an active form of the antagonist (Schoepp et al., 1990b) on $Ca^{2+}$ mobilization induced by quisqualate (1 μM), L-glutamate (100 μM), ibotenate (300 μM), and *trans*-ACPD (100 μM) in cells on days 1–27 of culture. Slight blockade was observed in only 6 of 80 cells. This blockade was independent of the culture period (one cell each on days 1, 16, and 21 and three cells on day 5 of culture) and of the type of cells (three Purkinje cells and three non-Purkinje cells including one Q-type cell). It was difficult to distinguish the apparent partial blockade observed in the six cells from deterioration of the cells because the blockade was not reversed by washing the cells for 10 min. It is clear, however, that most of the cerebellar neurons that showed $Ca^{2+}$ mobilization were not blocked by AP3.

We then examined W7 and TMB-8, drugs used to inhibit $IP_3$-induced $Ca^{2+}$ release (IICR) (Hill et al., 1988; Palade et al., 1989). In Purkinje cells, W7 (50 μM) and TMB-8 (500 μM) blocked quisqualate-induced $Ca^{2+}$ mobilization in a reversible manner while blocking the KCl-induced $Ca^{2+}$ elevation to a lesser extent (Fig. 3*A*). W7 at 20 μM and TMB-8 at 200 μM showed only partial blockade, indicating dose-dependent blocking actions. W5 (50 μM), an analog and weak calmodulin inhibitor, was not inhibitory. These results again indicated that

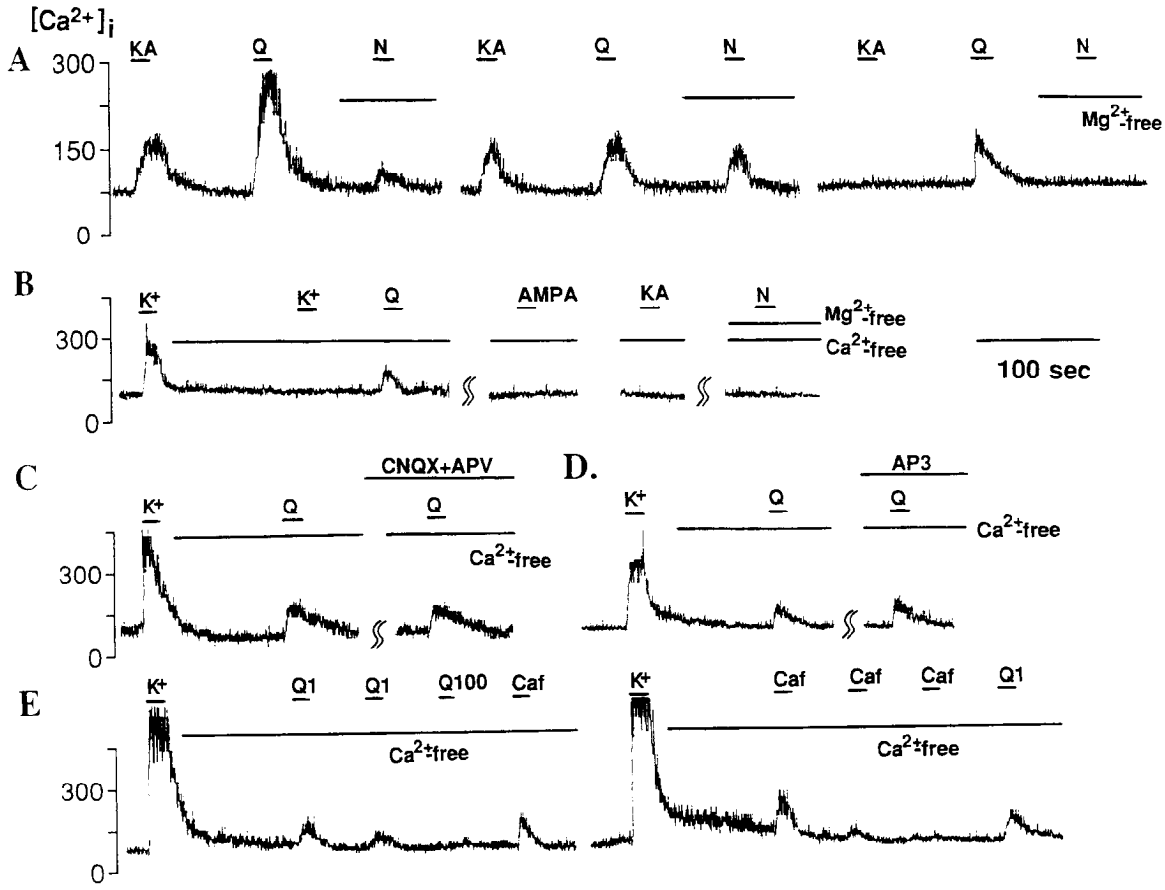



*Figure 2.* $Ca^{2+}$ influx and mobilization from intracellular stores by EAAs. *A*, Typical responses of cerebellar neurons to kainate (*KA*; 10 μM), quisqualate (*Q*; 1 μM), and NMDA (*N*; 100 μM) in medium containing $Ca^{2+}$. Immunocytochemically identified Purkinje cells showed characteristic response patterns (*left trace*). Non-Purkinje cells were classified according to the patterns of their responses, as those that were sensitive to kainate, quisqualate, and NMDA (*middle trace*; "typical" response) and those that were sensitive to only quisqualate (*right trace*; "Q-type" response). *B*, Quisqualate (1 μM), but not AMPA (100 μM), kainate (20 μM), or NMDA (100 μM), induced increase in $[Ca^{2+}]_i$ in $Ca^{2+}$-free solution. The insensitivity to high-$K^+$ (50 mM)–induced depolarization confirmed that the $Ca^{2+}$ concentration in the solution was low after washing the cells with $Ca^{2+}$-free medium for 90 sec. AMPA, kainate, and NMDA were also applied after high-$K^+$ stimulation (traces not shown; see Materials and Methods). The trace shown was obtained from a Purkinje cell on day 9 of culture and is representative of 10 Purkinje and 22 non-Purkinje cells (18 typical and 4 Q-type cells). *C*, A combination of CNQX (50 μM) and D,L-APV (200 μM) did not block quisqualate (1 μM)-induced $Ca^{2+}$ mobilization. The trace is from a Purkinje cell on day 16 of culture and is representative of five Purkinje and eight non-Purkinje cells (six typical and two Q-type cells). *D*, D,L-AP3 (1 mM) was ineffective in blocking $Ca^{2+}$ mobilization by quisqualate (1 μM) in a Purkinje cell on day 4 of culture. The trace is representative of 74 of 80 cells tested on different culture days. Six cells showed partial irreversible blockade by 2 mM L-AP3 (see Results). *E*, After the cell became refractory to 100 μM quisqualate by repeated application of 1 μM quisqualate, caffeine (*Caf*; 10 mM) induced $Ca^{2+}$ mobilization (*left trace*). Quisqualate (1 μM) induced $Ca^{2+}$ mobilization when the caffeine-sensitive pool was depleted by repeated application of 10 mM caffeine (*right trace*). The *left trace* was from a Purkinje cell on day 5 of culture and is representative of two Purkinje and four non-Purkinje cells. The *right trace* was from a Purkinje cell on day 4 and is representative of one Purkinje and two non-Purkinje cells.

$Ca^{2+}$ mobilization by quisqualate was mediated by mGluR coupled to IICR. It should be noted, however, that W7 and TMB-8 also blocked CICR in cells that responded to the repeated application of caffeine. Similar results were obtained in all types of non-Purkinje cells and are summarized in Figure 3*B*. The metabotropic responses in non-Purkinje cells were pharmacologically indistinguishable from those in Purkinje cells.

*Dose–response relationship of the mGluR*

The dose–response relationship of $Ca^{2+}$-mobilizing responses against mGluR agonists studied at single Purkinje cells revealed an all-or-none tendency, as shown most typically in Figure 4*A*. The tendency was masked when the responses were averaged over many cells (Fig. 4*B*) because the thresholds of individual cells varied widely, being 0.03–1 μM for quisqualate, 3–100 μM for L-glutamate, 30–100 μM for ibotenate, and 30–500 μM for *trans*-ACPD. In the absence of extracellular $Ca^{2+}$, the mGluR agonists showed the following rank order of potency: quisqualate > glutamate > ibotenate ≅ *trans*-ACPD. A similar dose–response relationship was obtained for non-Purkinje cells except that the KCl-insensitive Q-type cells (Fig. 2*A*, right trace) showed more prominent all-or-none responses (not shown).

L-Aspartate, which is one of the candidates of the endogenous transmitter at climbing fiber–Purkinje cell synapses, had no effect on this metabotropic receptor even at 1 mM (Fig. 4*B*), suggesting that the mGluRs in Purkinje cells are not activated by climbing fibers.

The existence of subtypes of mGluRs with preference for ibotenate and quisqualate, respectively, has been suggested (Sladeczek et al., 1988), but in our study, all neurons that showed a $Ca^{2+}$-mobilizing response to one of the mGluR agonists responded to the other mGluR agonists in a similar fashion ($n$ =

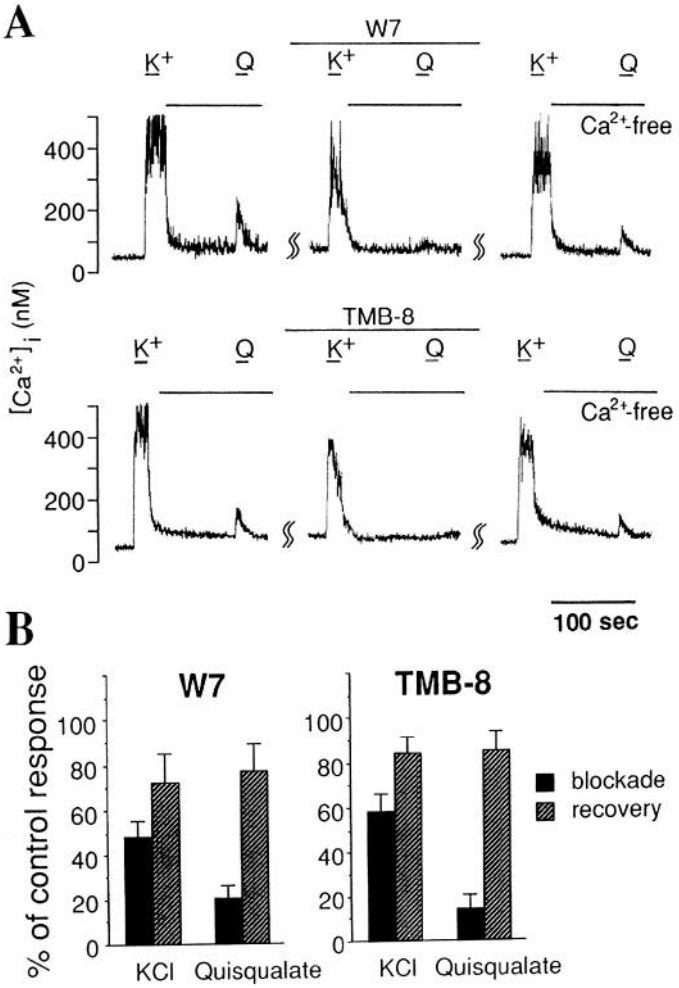



*Figure 3.* Blockade of $Ca^{2+}$-mobilizing response by inhibitors of IICR. *A*, After the control responses had been obtained (*left column*), the cells were incubated with inhibitors for 5 min and then tested with 50 mM KCl (*C*) and 1 μM quisqualate (*Q*; *middle column*) in the presence of the inhibitors. They were then washed for 10 min with medium and retested with KCl and quisqualate (*right column*) to determine the reversibility of the blockade. W7 (50 μM; *upper trace*) or TMB-8 (500 μM; *lower trace*) suppressed the quisqualate-induced $[Ca^{2+}]_i$ increases with less effect on KCl-induced responses. Traces are from a Purkinje cell on day 5 (*upper trace*) and day 8 (*lower trace*) of culture. *B*, Summary of results on blockade. The degree of blockade and recovery are expressed as percentages of the control response before application of antagonists. All cells showing metabotropic responses were blocked similarly by W7 and TMB-8. *Columns* and *bars* represent means ± SEM ($n$ = 18, including 7 typical and 2 Q-type non-Purkinje cells, for W7; $n$ = 9, including 1 typical and 1 Q-type non-Purkinje cell, for TMB-8).

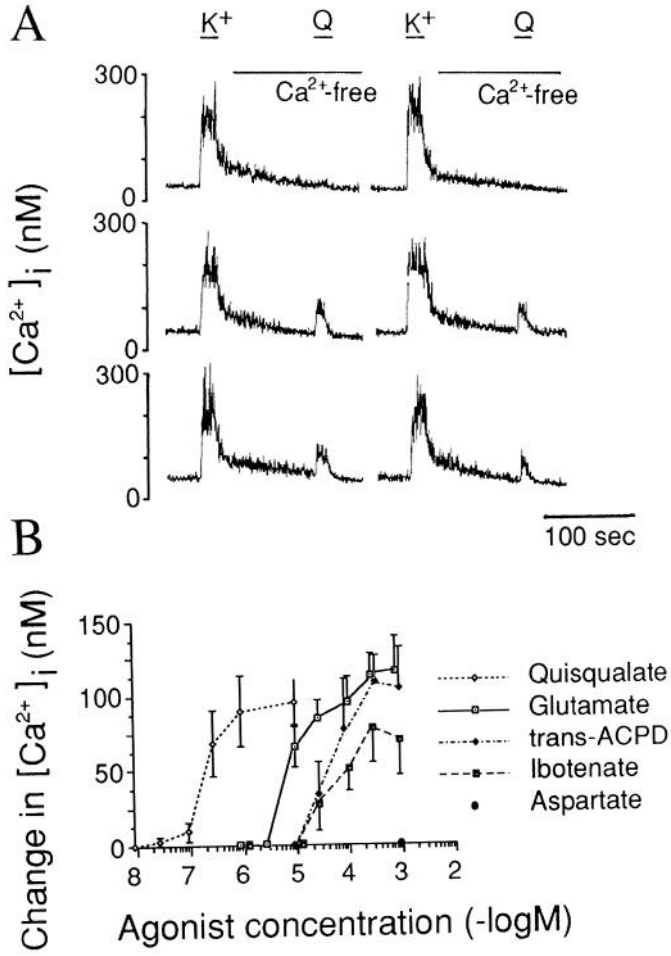



*Figure 4.* Dose–response relationship of metabotropic glutamate responses. *A*, Typical all-or-none responses to increasing doses of quisqualate in a Purkinje cell on day 5 of culture. Concentrations of quisqualate were 12.5, 25, 50, 100, and 200 nM and 1.6 μM, increasing from the *upper left to lower right trace*. *B*, Summary of averaged dose–response relationship in Purkinje cells. Neurons were exposed to increasing concentrations of the indicated agonists in $Ca^{2+}$-free medium. The average amplitudes of $Ca^{2+}$ mobilization are plotted against the log concentrations of the agonists. Values for quisqualate, glutamate, *trans*-ACPD, and ibotenate are means ± SEM for 10, 14, 5, and 6 Purkinje cells, respectively.

24, including 3 Purkinje cells, on day 2 of culture; $n$ = 27, including 4 Purkinje cells, on day 7). We therefore used quisqualate as a representative mGluR agonist in most experiments.

### *Developmental change of the metabotropic response to quisqualate*

The fraction of Purkinje cells exhibiting $Ca^{2+}$ mobilization in response to quisqualate changed markedly during development of cells in culture (Fig. 5*A*). The amplitudes of $Ca^{2+}$ mobilization in the responding cells also showed a similar developmental pattern (Fig. 5*B*), indicating that the functional $Ca^{2+}$-mobilizing machinery decreased in number in all Purkinje cells. On the other hand, the percentage of responding non-Purkinje cells and their amplitude of response remained fairly constant (Fig. 5*A,B*).

Figure 5*B* also shows the change in the responses to iGluR agonists during culture. The responses to NMDA showed an interesting developmental pattern: they developed later and diminished earlier than the responses to non-NMDA agonists in both Purkinje and non-Purkinje cells. This finding is consistent with results of electrophysiological experiments on cerebellar slices (Dupont et al., 1987; Garthwaite et al., 1987) and further supports the view that the neurons in our culture system differentiated in a manner similar to those *in vivo*.

### *Spatial distribution of $IP_3R$ and mGluR and their responses within single Purkinje cells*

To know whether the developmental change in $Ca^{2+}$ mobilization by quisqualate in Purkinje cell is caused by the loss or change in localization of mGluR and $IP_3R$, we visualized both receptors immunocytochemically. $IP_3R$ (Figs. 1, 6*A–C*) and

mGluR (Fig. 6*D,E*) proteins were recognized from as early as day 1 of culture and steadily increased thereafter. Non-Purkinje cells that showed $Ca^{2+}$ mobilization in response to quisqualate were hardly stained with anti-$IP_3R$ or anti-mGluR antibody, suggesting the presence of heterogeneity of the receptors in these cells. Both receptors were evenly distributed throughout the Purkinje cell including the somata and fine dendrites. All Purkinje cells gave a positive reaction for mGluR on day 25 in culture (Fig. 6*E*), when most of the $Ca^{2+}$-mobilizing reaction is lost (Fig. 5*A*). Thus, the intracellular receptors ($IP_3R$) and extracellular receptors (mGluR) for metabotropic response were expressed throughout the cell in all Purkinje cells at all stages of development.

We next analyzed the distribution of the $Ca^{2+}$-mobilizing response within single Purkinje cells using an image processor instead of measuring averaged $Ca^{2+}$ change within windows. As shown in Figure 7*A–C*, $[Ca^{2+}]_i$ elevation stimulated by high $K^+$ started from the entire surface and spread to the center of the soma of Purkinje cells on day 4 of culture. $Ca^{2+}$ changes can be observed at proximal neurites (Fig. 7, arrows). On the other hand, $Ca^{2+}$ mobilization induced by quisqualate in $Ca^{2+}$-free medium began in, and was mainly localized to, the center of the soma (Fig. 7*D–F*). Thus, the distribution of metabotropic responses does not match those of mGluR and $IP_3R$, indicating that some mGluR and $IP_3R$ are not functional even within Purkinje cells at an early developmental stage.

*Prevention of apparent mGluR desensitization*

Recently, Catania et al. (1991) showed that glutamate caused the desensitization of mGluR by activation of protein kinase C (PKC). It is possible that glutamate produced from neurons caused the desensitization of mGluR and loss of $Ca^{2+}$ mobilization during culture. Another possibility is that ADP-ribosylation of G-protein caused the apparent desensitization of mGluR, as endogenous ADP-ribosylation of G-protein is responsible for the decoupling of G-protein and PLC in primary cultures of hepatocytes (Itoh et al., 1984). We tested these possibilities by addition of polymyxin B (30 μg/ml) and sphingosine (10 μM) to block PKC, nicotinamide (25 mM) to block ADP-ribosyltransferase, and GABA (50 μM) to reduce the release of endogenous glutamate to the medium during culture. As summarized in Table 1, these treatments caused little change in the percentage of Purkinje cells exhibiting $Ca^{2+}$ mobilization to quisqualate or the amplitude of the responses on day 6 of culture. On day 15 of culture, Purkinje cells did not survive well in medium containing nicotinamide or sphingosine. The percentage and the amplitude of the metabotropic responses in GABA- or polymyxin B-treated Purkinje cells were similar to those in control cells. These results suggest that developmental change in metabotropic responses in Purkinje cells involves a mechanism other than phosphorylation by PKC or ADP-ribosylation.

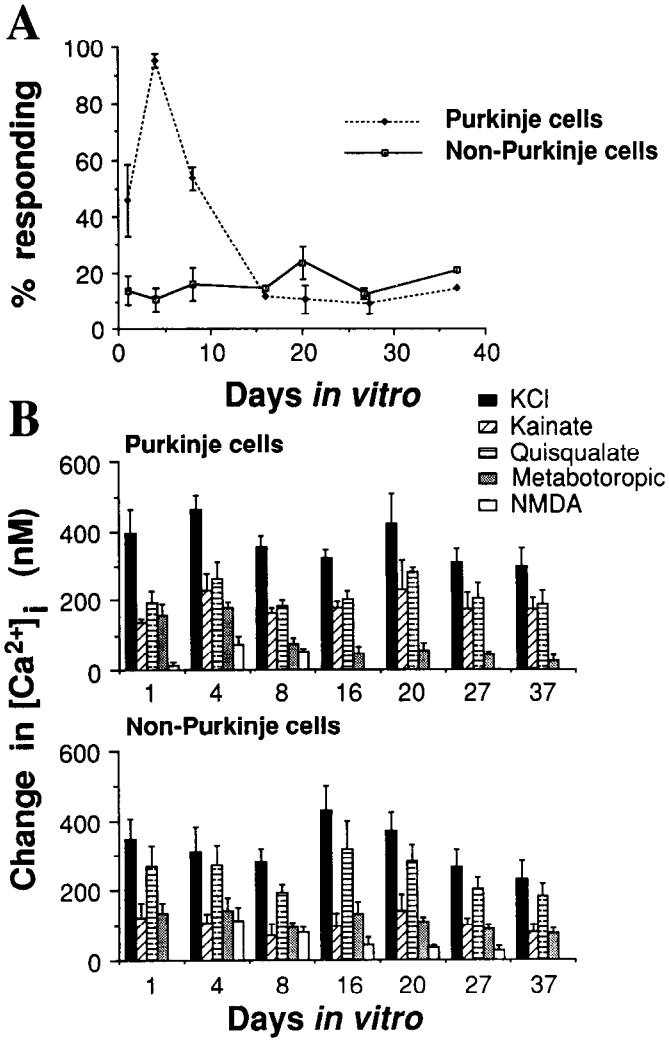


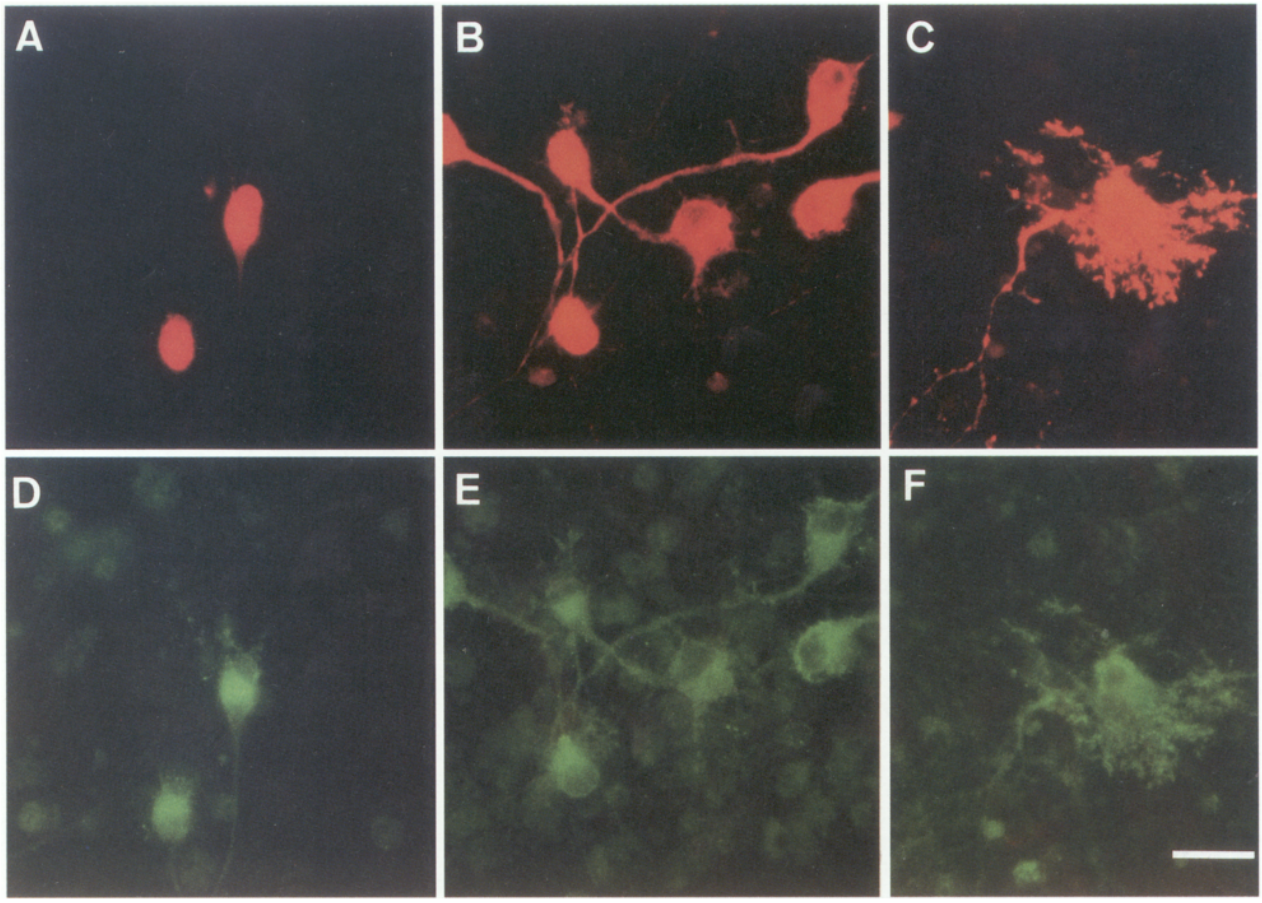

*Figure 5.* Developmental change of the quisqualate-induced $Ca^{2+}$-mobilizing response. *A*, Percentages of Purkinje (*broken line*) and non-Purkinje (*solid line*) cells exhibiting $Ca^{2+}$ mobilization in response to 5 μM quisqualate in $Ca^{2+}$-free medium are plotted against the days in culture. *B*, Amplitudes of $Ca^{2+}$ mobilization induced by 5 μM quisqualate in neurons showing a metabotropic response on various days of culture. The amplitudes of $[Ca^{2+}]_i$ increases in response to 50 mM KCl, 20 μM kainate, 5 μM quisqualate, and 100 μM NMDA in these cells in medium containing $Ca^{2+}$ are also shown for comparison with the amplitudes of the metabotropic responses. The responses to these agonists in neurons showing no metabotropic responses were similar in amplitude (not shown). Note that the responses to quisqualate in non-Purkinje cells are larger than those expected from the middle trace in Figure 2*A*. This is because the amplitudes of the responses to kainate and NMDA are averages for all non-Purkinje cells including Q-type cells. Data are means ± SEM from at least three independent cultures, except for those on days 16 and 37, which are for single cultures.

## Discussion

*mGluRs in cultured cerebellar neurons*

In this study, we showed that Purkinje neurons and some types of non-Purkinje neurons in primary culture exhibit $Ca^{2+}$-mobilizing responses to mGluR agonists. These responses were mediated by mGluR coupled to PI metabolism because they were not blocked by iGluR antagonists (Fig. 2*E*), were independent of the caffeine-sensitive pool (Fig. 2*C*), and were blocked by "IICR inhibitors" (Fig. 3). We characterized functional mGluR pharmacologically at single Purkinje cells for the first time. Their sensitivities to agonists and antagonists were different from those observed in earlier studies in which the responses of populations of cells were monitored. We also found that the metabotropic responses of Purkinje cells showed a unique developmental change in sensitivity, and investigated the cause of the change.

Analysis of receptor activity in dispersed cells in culture has several advantages over studies *in vivo* or with brain slices. Better control of the external environment of neurons enabled us to record the direct effects of drugs. Easy access to neurons and better oxygenation permitted more stable recording, so the ef-



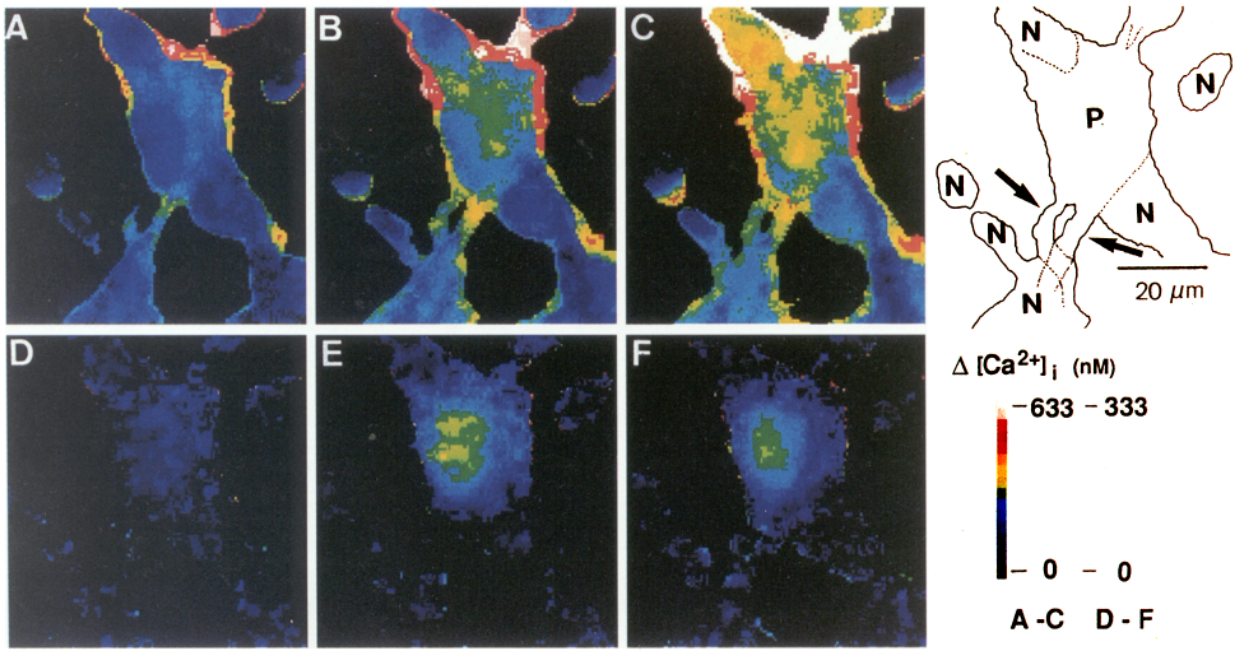




*Figure 6.* Distributions and developmental expressions of $IP_3R$ and mGluR proteins. Double-immunofluorescent staining used FITC-conjugated anti-rat secondary antibody to label $IP_3R$ (*A–C*) and TRITC-conjugated anti-rabbit secondary antibody to label mGluR (*D–F*). Bright stainings for $IP_3R$ and mGluR were colocalized in the same cells on days 4 (*A, D*), 10 (*B, E*), and 25 (*C, F*). The two stainings were distributed similarly throughout single Purkinje cells. Scale bar, 20 μm.

*Figure 7.* Digital image analysis of changes in $[Ca^{2+}]_i$ within an immature Purkinje cell. Changes in $[Ca^{2+}]_i$ were visualized spatially by digital subtraction of the fluorescence image before each stimulus. *A–C*, At 0.5, 1, and 3 sec after exposure to high $K^+$ (50 mM) in medium containing $Ca^{2+}$. *D–F*, At 0.5, 1, and 3 sec after exposure to quisqualate (1 μM) in $Ca^{2+}$-free medium. The outlines of the cells were traced (*right upper corner*) after immunological staining. The Purkinje cell (*P*) was surrounded by several non-Purkinje cells (*N*). *Arrows* indicate the neurites of the Purkinje cell. This cell was examined on day 4 of culture, and results are representative of five Purkinje cells examined. The $[Ca^{2+}]_i$ is presented as a scale in the *right lower corner*. Scale bar, 20 μm.

fects of drugs could be tested repeatedly. The mechanism of the change in receptor sensitivity during maturation is difficult to be explored unless a culture system is used.

There are, however, two major technical problems in use of cultured cells (see introductory remarks). With regard to the second problem of the possibility of abnormal differentiation in culture, the form and arrangement of the dendritic tree-like arborization of Purkinje cells in our cultures (Fig. 1) were not typical of fully differentiated Purkinje cells *in vivo*, but were more reminiscent of Purkinje cells in the *reeler* mutant that develop ectopically. This may have been due to the lack of extracerebellar afferents and a normal laminar structure. However, the developmental profiles of expression of $IP_3R$ and mGluR proteins in culture (Figs. 1, 6) were comparable to those *in vivo* (Nakanishi et al., 1991; Ryo, Miyawaki, Furuichi, and Mikoshiba, unpublished observations). Moreover, the developmental changes in sensitivities to NMDA and mGluR agonists of the Purkinje cells that we observed in culture have also been observed in fresh cerebellar slices from rats of different ages (Garthwaite et al., 1987; Palmer et al., 1990). Spontaneous synaptic activity, which is essential for the dendritic growth of Purkinje cells in culture (Schilling et al., 1991), was also observed in our cultures. Our culture system thus appeared valid for studies on mGluR in Purkinje cells.

Quisqualate-induced $Ca^{2+}$ mobilization has also been demonstrated in cultured single hippocampal neurons (Murphy and Miller, 1988, 1989; Furuya et al., 1989). However, in these previous studies, the relationship of this $Ca^{2+}$-mobilizing response to PI metabolism was unclear and the cells were not identified, so the observed responses may have included those of several types of cells (i.e., granule and pyramidal cells as well as interneurons from both the hippocampus and dentate gyrus).

*Pharmacology of mGluR in single neurons*

The dose–response relationship of the $Ca^{2+}$-mobilizing response in single neurons differed in several ways from that observed in earlier studies in which PI turnover of populations of cells was monitored in cerebellar slices (Blackstone et al., 1989) or in cultured granule cells (Nicoletti et al., 1986b). First, the cells responded to mGluR agonists in an all-or-none manner (Fig. 4*A*). PI turnover increases gradually with increase in the log concentration of agonists (Nicoletti et al., 1986b; Blackstone et al., 1989), indicating that the $IP_3R$ is fully activated by a relatively small suprathreshold dose of $IP_3$. A similar all-or-none response of the $IP_3R$ has been observed in single hepatocytes (Ogden et al., 1990), and may be explained by $Ca^{2+}$ sensitization of $IP_3R$ (Missiaen et al., 1991). Second, glutamate had a more potent effect in the present study (Fig. 4*B*) than in slices. This can be explained by the abundance of astrocytes that take up glutamate in slices. Third, L-aspartate, even at 1 mM, did not evoke any $Ca^{2+}$ mobilization in the present study, but induced PI turnover in previous studies. This PI turnover induced by L-aspartate was probably not due to the direct activation of mGluRs, but rather to increased PLC activity subsequent to $Ca^{2+}$ entry from the medium, as $Ca^{2+}$ was present in the medium in previous studies. Thus, the response relationship we obtained in the absence of extracellular $Ca^{2+}$ is probably more accurate.

The insensitivities of the metabotropic responses to AP3 and PTX can be explained by the all-or-none nature of these responses. The $Ca^{2+}$-mobilizing response is not blocked unless the production of $IP_3$ is reduced to a subthreshold level, but AP3 (Schoepp and Johnson, 1989) and PTX (Nicoletti et al., 1988; Ambrosini and Meldolesi, 1989) suppressed PI turnover to only about 70–80% of the control level. The insensitivity of mGluR to AP3 cannot be attributed to their developmental change (Nicoletti et al., 1986a) or to the agonist used to elicit the metabotropic response (Tanabe et al., 1991), because even high doses of the active forms of AP3 did not completely block the metabotropic responses induced by any mGluR agonist tested at any developmental stage of the neurons in our study. An alternative possibility that mGluRs in cerebellar neurons are coupled to PTX-insensitive G-protein and are of the AP3-insensitive type is less likely, at least in Purkinje cells, because mGluR1 is the predominant subtype expressed in Purkinje cells (Tanabe et al., 1992).

**Table 1. Effect of drugs on desensitization of $Ca^{2+}$ mobilization induced by quisqualate (1 μM) in Purkinje cells during development**

| | Responsive Purkinje cells (%) | Amplitude of $Ca^{2+}$ change (nM) | Number of Purkinje cells |
|---|---|---|---|
| Six days *in vitro* | | | |
| Control | 61.5 | 76 ± 5 | 26 |
| Polymyxin B (30 μg/ml) | 63.2 | 68 ± 7 | 19 |
| Sphingosine (10 μM) | 62.5 | 66 ± 7 | 16 |
| Nicotinamide (25 mM) | 56.3 | 67 ± 6 | 16 |
| GABA (50 μM) | 65.4 | 83 ± 6 | 26 |
| Fifteen days *in vitro* | | | |
| Control | 12.5 | 43 ± 2 | 16 |
| Polymyxin B (30 μg/ml) | 13.3 | 46 ± 3 | 15 |
| GABA (50 μM) | 14.3 | 42 ± 4 | 14 |

Values are means ± SEM.

The site of action of W7 and TMB-8 has been suggested to be the channel portion of the $IP_3R$–channel complex because these antagonists do not compete with $IP_3$ for binding to its receptor (Hill et al., 1988; Palade et al., 1989). We consider that the inhibitory effect of W7 was caused by direct interactions with calmodulin because W5, an analog and weak calmodulin inhibitor, was not inhibitory and at the low dose of W7 used, it was unlikely to have a nonspecific effect on other kinases. The demonstration of calmodulin-binding sites in the $IP_3R$–channel complex (Maeda et al., 1991) is of much interest in this respect. Some blocking effect by W7 on KCl-induced elevation in $[Ca^{2+}]_i$ may be due to the blockade of $Ca^{2+}$ channels associated with calmodulin (Johnson, 1984; Phillips et al., 1992). TMB-8 and W7 may also have inhibited the responses to KCl by blocking the KCl-induced PI turnover (e.g., Baudry et al., 1986). Although W7 and TMB-8 may not in strictness be considered as specific blockers of IICR, they are the only reversible blockers of metabotropic responses available at present and will be useful to study mGluR functions in neurons.

*Developmental change of mGluR in Purkinje cells*

The percentage of Purkinje cells exhibiting metabotropic responses to quisqualate changed markedly during development, with a sharp peak after day 4 of culture (Fig. 5*A*). This is in sharp contrast with findings in non-Purkinje cells, although the pharmacological characteristics of mGluR in non-Purkinje cells were indistinguishable from those in Purkinje cells. The PI metabolism stimulated by quisqualate in cerebellar slices showed a very similar pattern of developmental change with a peak on postnatal day 6 (Palmer et al., 1990). In addition to confirming

this result of Palmer et al., we demonstrated that these changes mainly occurred in Purkinje cells.

As $Ca^{2+}$ mobilization by quisqualate is due to a cascade of reactions involving mGluR, G-protein, PLC, $IP_3R$, and $Ca^{2+}$ stores, decrease in the functional $Ca^{2+}$-mobilizing machinery reflects absolute or functional loss of one or more of the components of the cascade. We consider that functional loss of the metabotropic responses was due to posttranslational modification of mGluR, G-protein, or PLC because (1) PI turnover declines during development (Palmer et al., 1990), indicating that a component before $IP_3$ production is impaired; (2) mRNAs for PLC are transcribed well in adult Purkinje cells (Ross et al., 1989); and (3) mGluR and $IP_3R$ were demonstrated immunocytochemically in Purkinje cells at various developmental stages (Fig. 6). In the future, the G-protein coupling to PLC should be identified and PLC protein in addition to its mRNA in Purkinje cells should be demonstrated.

We demonstrated the metabotropic response in young Purkinje cells for the first time (Fig. 7) and found that some mGluR and $IP_3R$ are not functional even in young Purkinje cells. This suggests that functional expression of the $Ca^{2+}$-mobilizing machinery is spatially as well as developmentally controlled in young Purkinje cells.

We investigated these developmental changes in metabotropic responses in somata of Purkinje cells (see Materials and Methods). $Ca^{2+}$ mobilization in fine dendrites was difficult to monitor because the volume was small and the Purkinje cells were often surrounded by many non-Purkinje cells that concealed the fluorescence image of neurites (compare Fig. 1). Recently, Llano et al. (1991), using cerebellar slices of 17–33-d-old rats, showed that quisqualate induces $Ca^{2+}$ mobilization not in somata but in dendrites of Purkinje cells. Thus, the location of functional mGluR may shift from soma, where we routinely measured $Ca^{2+}$ mobilization responses, to dendrites of Purkinje cells during maturation. The absence of metabotropic responses in soma of Purkinje cells (Llano et al., 1991) is consistent with the idea that most of the $IP_3R$ and mGluR proteins expressed are not functional at a late developmental stage.

Inhibition of PKC or ADP-ribosyltransferase did not prevent decrease in the $Ca^{2+}$-mobilizing responses in Purkinje cells during culture (Table 1). This indicates that the apparent desensitization of mGluR during maturation cannot be attributed simply to the mechanism in short-term desensitization of metabotropic glutamate responses (Catania et al., 1991) or of adrenergic responses (Itoh et al., 1984). Possibly other kinases are involved. At the same time, it is possible that inhibition of endogenous PKC activity and ADP-ribosylation during culture may have affected other neuronal functions and masked some preventive effects of these drugs on the desensitization. This possibility is supported by the poor survival of inhibitor-treated neurons on day 15 of culture (Table 1), but the neurons were morphologically and pharmacologically similar to control ones on day 6. Short-term exposure to phosphatase, instead of long-term exposure to kinase inhibitors, may be helpful for further clarification of the involvement of phosphorylation.

The transient response of Purkinje cells to NMDA has been considered to be associated with synaptogenesis as NMDA stimulated neurite outgrowth from granule cells in culture (Pearce et al., 1987). NMDA receptors may cause growth stimulation due to their high permeability to $Ca^{2+}$, which is important for neurite extension (Connor, 1986). Interestingly, the NMDA receptor is not functional in adult Purkinje cells although its mRNA is expressed (Moriyoshi et al., 1991). The high $Ca^{2+}$-mobilizing responses in Purkinje cells during the early developmental period thus suggest a similar role of mGluRs in establishing synaptic contacts. In addition, the dynamic change in the functional $Ca^{2+}$-mobilizing machinery may serve as a synaptic modification process such as in LTD or LTP. Further investigation of the mechanism of desensitization of metabotropic responses is therefore of much importance, and for this purpose our culture system should be useful.

*Note added in proof.* We stated in the Discussion that the possibility that mGluRs in cerebellar neurons are coupled to PTX-insensitive G-protein and are of the AP3-insensitive type is "less likely" since mGluR responses in *Xenopus* oocyte injected with mGluR1 mRNA or cerebellar mRNA had been shown to be sensitive to PTX and D,L-AP3. After the submission of this work, however, Aramori and Nakanishi reported that mGluR responses are insensitive to PTX and D,L-AP3 in CHO cells transfected with mGluR1 cDNA (*Neuron* 8: 757–765, 1992). It is thus possible that the insensitivities of the metabotropic responses to PTX and AP3 we observed in cerebellar neurons reflects the actual insensitivity of mGluR to AP3 and PTX in these neurons.

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