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Mechanisms Underlying Developmental Changes in the Expression of Metabotropic Glutamate Receptors in Cultured Cerebellar Granule Cells: Homologous Desensitization and Interactive Effects Involving *N*-Methyl-D-aspartate Receptors

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SUMMARY

Glutamate receptors coupled to polyphosphoinositide (PPI) hydrolysis (metabotropic glutamate receptors, mGluR), are highly efficient during the early stages of postnatal life and are thought to be involved in developmental plasticity. The dramatic decrease with age in mGluR activity suggests the existence of mechanisms that down-regulate this receptor after a certain stage of neuronal maturation. In cultured cerebellar granule neurons grown under conditions that promote the survival and maturation of cells (serum-containing medium with 25 mM K^+), enzymatic depletion of extracellular glutamate prevented the age-dependent decrease in mGluR agonist-stimulated PPI hydrolysis that normally occurs after 4 days of maturation *in vitro*, suggesting that mGluR activity declines as a result of developmental changes affecting homologous desensitization. This was borne out by the observation that glutamate at low concentrations (1–10 μM) readily desensitized mGluR at 7 days but not at 4 days in culture. Furthermore, the critical period during which the high sensitivity to agonist-induced desensitization of mGluR developed coincided with the period when phorbol ester-activated protein kinase C acquired the ability to suppress mGluR activity.

The developmental pattern of mGluR agonist-induced PPI hydrolysis was similar in granule cells grown under "trophic" and "nontrophic" conditions (in cultures in 25 mM K^+ and in a medium containing "low" K^+, in this study, 10 mM, respectively). However, the developmental decline in the response to mGluR stimulation after 4 days *in vitro* was not prevented in cells grown in 10 mM K^+ by the removal of extracellular glutamate; rather, it could be counteracted by treatment with *N*-methyl-D-aspartate (NMDA) (EC_{50}, approximately 4 μM), which blocked the development of mGluR desensitization. The effect was NMDA receptor mediated and required DNA transcription and protein synthesis. However, NMDA exerted a different effect in cells grown in 25 mM K^+, inducing a substantial decrease rather than an increase in mGluR activity. The effect of growth conditions was also examined on mGluR mRNA levels, which were not always correlated with mGluR activity. In general, either increases in the medium K^+ concentrations or NMDA supplementation of the cultures resulted in a decrease in mGluR mRNA levels.

It is noteworthy that NMDA could also restore mGluR activity after the metabotropic response had reached its peak. This implies that NMDA receptor activation may be involved in the increase in mGluR activity in adult life under conditions that elicit plastic changes in the nervous system.

mGluR forms a family of G protein-coupled receptors associated with multiple signal transduction pathways. At least two receptor subtypes, mGluR1 and mGluR5, are coupled to PPI hydrolysis, whereas some subtypes are linked to adenylate cyclase (1). In the brain, the functional expression of $mGluR_{PI}$ is developmentally regulated, being maximal during early postnatal life and declining progressively with maturation to the adult level (2). In the adult, increased $mGluR_{PI}$ activity can be induced by a variety of conditions, including deafferentation (3), electrical kindling (4, 5), the formation of LTP of excitatory synaptic transmission (6), the acquisition of spatial learning (7), and ischemic neuronal damage (8, 9).

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ABBREVIATIONS: mGluR, metabotropic glutamate receptor; PPI, polyphosphoinositide; $mGluR_{PI}$, PPI hydrolysis-coupled mGluR; LTP, long-term potentiation; PKC, protein kinase C; EAAs, excitatory amino acids; NMDA, *N*-methyl-D-aspartate; InsP, [^{3}H]inositol monophosphate; SSC, standard saline citrate; [^{3}H]PdBu, 4-β-[^{3}H]phorbol-12,13-dibutyrate; ACPD, (1*S*, 3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclohepten-5,10-imine maleate; AlAT, alanine amino transferase; CCh, carbamylcholine; AMPA, amino-3-hydroxy-5-methyl-4-isoxazole propionate; AP5, D-2-amino-5-phosphonopentanoate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

The activation of $mGluR_{PI}$ results in the generation of the second messengers inositol triphosphate and diacylglycerol, which are involved in the mobilization of Ca^{2+} from intracellular stores and the activation of PKC, respectively. Elevated cytoplasmic Ca^{2+} and activated PKC have profound and often long-lasting effects on many cellular properties (10). The early development of $mGluR_{PI}$ activity may play an important role by providing the Ca^{2+} transients and the means for PKC activation at a stage when other relevant cellular processes are not yet mature (see Ref. 11 and references therein). Despite recent progress in the characterization of mGluR (1), little is known about the mechanisms involved in the regulation of $mGluR_{PI}$ activity in nerve cells during development. We have studied the regulation of mGluR using cultures derived from early postnatal (P6–8) rat cerebella since this preparation offers a number of experimental advantages. These include having a largely homogeneous cellular composition—about 90% of the cells are granule neurons—and a more or less synchronous maturation of cells *in vitro* (12). In addition, cerebellar granule cells are endowed with functionally active $mGluR_{PI}$ (e.g., Ref. 13), and they express mRNA coding for mGluR1α (1, 11, 14).

Cerebellar granule cells in culture develop specific survival and maturation requirements, which can be met either by K^+-induced membrane depolarization (>20 mM K^+) or by treatment with EAA; NMDA is particularly effective (12). We reported that when cultures are grown under conditions that promote survival and maturation of granule cells (medium containing 10% fetal calf serum and 25 mM K^+), mGluR agonist-induced PPI hydrolysis increases sharply to a peak at 4 days *in vitro*, followed by a progressive decrease (11). This developmental pattern of activity proved to be positively correlated with changes in mGluR1α mRNA levels (11). It is also known that preexposure of granule cells to mGluR agonists reduces both the functional activity of $mGluR_{PI}$ and the expression of mGluR1 mRNA (15, 16). A decrease in the receptor activity can also be induced by depolarizing K^+ concentrations (16, 17), which stimulate glutamate release as a function of the maturation of cultured granule cells (18).

We now report that extracellular glutamate is critical for the age-dependent decline in the activity of $mGluR_{PI}$ under standard conditions (in 25 mM K^+) and that receptor desensitization is developmentally regulated, with high sensitivity manifest only after $mGluR_{PI}$ activity has reached its peak (*viz.* after 4 days *in vitro*). In addition, both the functional expression of $mGluR_{PI}$ and mGluR1α mRNA levels are subject to regulation in granule cells by environmental factors, in particular, by the concentration of extracellular K^+ and by NMDA exposure.

Materials and Methods

Preparation of cultured cerebellar neurons. Primary cultures of cerebellar granule cells were prepared from 8-day-old rats as described previously (19). Briefly, cerebella were sliced (450 × 450 μm), and the tissue was dissociated after trypsinization (0.025% trypsin solution, 37°, 15 min) and trituration in the presence of DNase (0.01%) and trypsin inhibitor (0.05%). Isolated cells were collected by centrifugation, resuspended in basal Eagle's medium (GIBCO, Grand Island, NY) containing 10% fetal calf serum, and seeded at a density of 2–2.5 × 10^5 cells/cm^2 onto 35-mm diameter Nunc Petri dishes (precoated with 10 μg/ml of poly-L-lysine). The culture medium also contained 2 mM glutamine (but no antibiotics), and the K^+ concentration was adjusted to either 10 or 25 and 40 mM as indicated. If not specified otherwise, NMDA was added once at 2 days *in vitro*. The medium was not changed during the experimental period. The growth of nonneuronal cells was inhibited by the addition of cytosine β-D-arabinofuranoside (10–20 μM) approximately 19 hr after seeding. These cultures contain >90% granule cells, 4–6% GABAergic neurons, and a small number (2–3%) of glial and endothelial cells (19).

Measurement of PPI hydrolysis. Inositol phospholipid hydrolysis was estimated in cultured cerebellar granule cells prelabeled with [^{3}H]inositol by measuring the accumulation of InsP in the presence of Li^+, as described previously (11). Briefly, cultures were labeled with 2 μCi/ml of *myo*-[2-^{3}H]inositol (specific activity, 16.5 Ci/mmol; Dupont-NEN) for 24 hr and then washed extensively with prewarmed Krebs-Henseleit buffer (equilibrated with 95% O_2/5% CO_2 to pH 7.4) containing 10 mM LiCl. Cells then were incubated in the same buffer at 37° in 95% O_2/5% CO_2 atmosphere in the presence or absence of the different agents tested. After 30 min, the buffer was replaced with ice-cold methanol/water (1:1), and the culture dishes were placed on dry ice. Cells then were harvested and added to a mixture of methanol, water, and chloroform (to give a final proportion of 1:1:1). [^{3}H]InsP, extracted in the aqueous phase, was separated by ion exchange chromatography and measured as described previously (11). Protein was estimated as described (11). $mGluR_{PI}$ desensitization was studied by pre-exposing cultures to glutamate or quisqualate during the labeling period with *myo*-[^{3}H]inositol. Cultures then were extensively washed and challenged with quisqualate for stimulation of PPI hydrolysis (15).

RNA isolation, electrophoresis, and hybridization. RNA was extracted from cultured cerebellar granule cells as described previously (11). Briefly, total RNA (about 20 μg/sample) was electrophoresed through 1.1% agarose and 2.2 M formaldehyde, stained with ethidium bromide, photographed under UV light, and then blotted onto nitrocellulose (Hybond-C extra, Amersham). Prehybridization (4–6 hr) and hybridization (18–24 hr) were carried out at 42° in a mixture containing 50% of formamide, 5× SSC (0.15 M NaCl and 0.015% sodium citrate), 5× Denhardt's solution, 20 mM sodium phosphate buffer (pH 6.5), and 100 μg of denaturated sheared salmon sperm DNA/milliliter. For hybridization, the mixture was supplemented with 10% of dextran sulfate and the labeled denaturated probe. The mGluR1α cDNA probe (pmGR1) was linearized using *Eco*RI and *Bam*HI, respectively, and ^{32}P labeled by the random primed DNA labeling method as described in Ref. 11. Nitrocellulose membranes were washed three times, first at room temperature (5 min each) and then at 52° (30 min each), with a mixture containing 2× SSC and 0.1% sodium dodecyl sulfate. Finally, the membranes were exposed to x-ray films at −70°, using intensifying screens. For quantitative evaluation, the autoradiograms were scanned using an LKB Ultroscan XL laser densitometer. The amount of ribosomal RNA (28 S and 18 S) was also determined by densitometry, analyzing the photographic negative (Polaroid type 55 positive/negative black and white film) of the ethidium bromide-stained gel. Under the experimental conditions, the densitometric signal was a linear function of the amount of RNA loaded.

Measurement of glutamate levels. The glutamate concentration in the culture media was estimated at different days *in vitro*. The samples were analyzed with an amino acid analyzer (Chromakon) using a cation exchange column (200 × 4.6 mm), gradient elution, and fluorescence detection (*o*-phthaldialdehyde) (20).

Statistical analysis. Statistical analysis was carried out using either Student's *t* test (when two groups were compared) or one-way analysis of variance, followed, if the overall effect was significant ($p < 0.05$), by Newman-Keuls' test for multiple comparisons ($p = 0.05$). Data expressed as a percentage of agonist-induced [^{3}H]InsP formation were analyzed using the nonparametric Kruskal-Wallis test.

Materials. *myo*-[2-^{3}H]Inositol (specific radioactivity, 16.5 Ci/mmol), [α-^{32}P]deoxycytidine 5′-triphosphate (specific radioactivity 3000 Ci/mmol), and [^{3}H]PdBu (specific radioactivity, 12.5 Ci/mmol) were purchased from Dupont-NEN; quisqualate, ACPD, and DNQX were obtained from Tocris Neuramin (Bristol, United Kingdom). All the other drugs or chemicals were from Sigma (St. Louis, MO), except MK-801, which was a gift from Merck Sharpe and Dohme Research

Laboratories, and the pmGR1 cDNA probe, constructed by Dr. Masayuki Masu, which was a generous gift from Prof. S. Nakanishi (Institute for Immunology, Kyoto University, Japan).

Results

Extracellular levels of glutamate

Glutamate concentration in the culture medium containing 10% fetal calf serum was more than 50 μM before plating (Table 1). In cultures grown in 25 mM K^+, glutamate levels decreased by more than 90% within 24 hr after plating (1 day *in vitro*) and then stabilized—as a result of continuous release from and uptake by the cells—at about 2 μM during the period of 3 to 7 days *in vitro*. To deplete extracellular glutamate, 25 mM K^+ cultures were supplemented with AlAT (12.5 units/ml) and a great excess of pyruvate (5 mM) (21). We established that exogenous AlAT activity was maintained during the experimental period, but pyruvate was actively used by the cultures. Therefore, AlAT was added once at 4 days *in vitro*, whereas supplementation with pyruvate was repeated daily. Under these conditions, glutamate concentrations in the medium of the transaminase-treated cultures fell below the detection level (Table 1). In comparison with K25 cultures, steady-state levels of extracellular glutamate were reduced in the K10 (<1 μM) and elevated in K40 cultures at 7 days *in vitro* (Table 1).

Developmental changes in agonist-stimulated PPI hydrolysis in cultures grown under different conditions

Effect of extracellular K^+ concentration. When cultures were grown under standard conditions, in 25 mM K^+ medium, stimulation of PPI hydrolysis by concentrations of quisqualate that elicit maximal effects (100 μM; Ref. 13) increased sharply to a peak at 4 days *in vitro* and then declined progressively (see also Ref. 11). Continuous removal of extracellular glutamate by treatment with AlAT plus pyruvate prevented the developmental decline in quisqualate-stimulated PPI hydrolysis in 25 mM K^+ cultures (Fig. 1A). In cells grown in a medium containing 40 mM K^+, which promotes cell survival as well as 25 mM K^+ (22), $mGluR_{PI}$ activity was severely depressed (Fig. 1A).

In 10 mM K^+ cultures, the developmental profile of quisqualate-induced PPI hydrolysis was similar to that in cells grown in the 25 mM K^+ medium (Fig. 1B). However, the mechanisms underlying the decrease in $mGluR_{PI}$ activity after 4 days *in vitro* under these two conditions must have been different since in contrast to 25 mM K^+ cells, AlAT treatment had no influence on the developmental decline in 10 mM K^+ cultures (Fig. 1B).

We also examined developmental changes in the activity of the muscarinic cholinergic receptor, which is also coupled to PPI hydrolysis. Fig. 1C shows that CCh-induced PPI hydrolysis is different in cultures grown in "low" and "high" K^+-containing media; in 10 mM K^+, the muscarinic response decreases after 4 days *in vitro*, whereas in 25 mM K^+, it increases to a plateau by approximately 8 days *in vitro*. The stimulation of PPI hydrolysis by CCh was similar in cells cultured in 40 and 25 mM K^+ medium for 7 days (Fig. 1C).

Effect of treatment with EAA receptor agonists. In 10 mM K^+ cultures, not only quisqualate- but also CCh-induced PPI hydrolysis was reduced after 4 days *in vitro* (Fig. 1, B and C). Such an effect may be the result of suboptimal growth conditions, which can be improved by NMDA supplementation (23). The age-dependent decline in $mGluR_{PI}$ activity was completely prevented when cultures grown in 10 mM K^+ were chronically exposed to NMDA from 2 days *in vitro* (Fig. 1B and Table 2). EAA receptor agonists such as kainate and AMPA can also exert trophic influences on developing granule cells, but compared with NMDA, their efficacy is lower (12, 24, 25). Table 2 shows that the influence of chronic exposure to NMDA on $mGluR_{PI}$ activity was mimicked by kainate—albeit the prevention of the decline was less—but not by AMPA.

Treatment with NMDA affected quisqualate-stimulated PPI hydrolysis in a concentration-dependent manner (EC_{50}, approximately 4 μM NMDA). The effect of NMDA (100 μM) was prevented by the selective antagonist AP5 (160 μM) or MK-801 (1 μM) (supplementation of cultures with these substances at 4 days *in vitro* and quisqualate-induced [^{3}H]InsP formation determined at 6 days *in vitro*; data not shown).

To characterize further the effect of NMDA treatment on the $mGluR_{PI}$ activity in 10 mM K^+ cells, we added NMDA to the cultures at different times before measurement of quisqualate-stimulated PPI hydrolysis at 7 days *in vitro*. Fig. 2 shows that NMDA treatment resulted in an increase in quisqualate-induced PPI hydrolysis when applied not only before but also after the peak of mGluR activity had been reached. Although NMDA was ineffective when added 1 or 6 hr before testing $mGluR_{PI}$ activity (data not shown), its influence was evident after a delay of about 24 hr. Agents that interfere with *de novo* protein formation by inhibiting RNA or protein synthesis (actinomycin D, 0.5 μg/ml, or cycloheximide, 10 μg/ml) blocked the effect of NMDA treatment in restoring quisqualate-induced PPI hydrolysis toward the peak activity. In these experiments, 100 μM NMDA and the inhibitors were added to 10 mM K^+ cultures at 4 days *in vitro*, and mGluR activity was determined at 6 days *in vitro* after we observed that a 48-hr exposure to these compounds is well tolerated by the cultures. $mGluR_{PI}$ activity was not influenced by exposure to cycloheximide or actinomycin D in cultures that were not treated with NMDA (data not shown).

Treatment of 10 mM K^+ cultures with high concentrations of NMDA (100 μM) for 7 days increased PPI hydrolysis induced not only by quisqualate but also by CCh (Fig. 1C and Table 3). However, although a low concentration of NMDA (5 μM) remained effective in increasing quisqualate-evoked PPI hy-

TABLE 1

Medium glutamate concentrations in granule cell cultures under different conditions

Values are mean of duplicate determinations of triplicate medium samples from three different cultures. Glutamate levels were reduced by AlAT (12.5 units/ml plus 5 mM pyruvate added at 4 days *in vitro*; supplementation with pyruvate was repeated daily).

Conditions	Days *in vitro*	Glutamate
		μM
25 mM K^+	0	63.5 ± 0.5
	1	4.1 ± 0.1
	2	3.2 ± 0.5
	3	2.0 ± 0.1
	4	2.0 ± 0.1
	7	2.0 ± 0.1
25 mM K^+ + AlAT	7	ND[a]
10 mM K^+	7	<1[b]
40 mM K^+	7	4.0 ± 0.2

[a] ND, not detectable; [b] In one culture, the concentration was 1 ± 0.01 μM, and in two other cultures, the amount of glutamate was below the detection level.

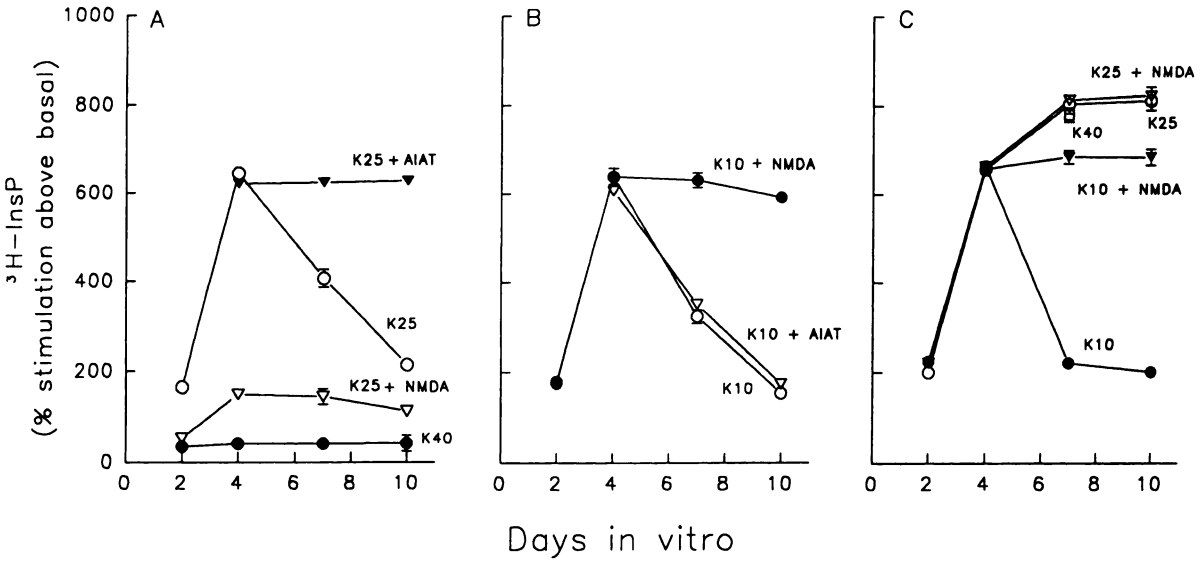

Fig. 1. [^{3}H]InsP formation induced by quisqualate (A and B) or carbamylcholine (C) (each at 100 μM) during the development of cerebellar granule cells grown in media containing different concentrations of K^+ (mM: 25, 40 in A and 10 in B). When indicated, cultures were treated with 100 μM NMDA (added at 2 days *in vitro*) or with AIAT (12.5 units/ml plus 5 mM pyruvate added at 4 days *in vitro*; supplementation with pyruvate was repeated daily). Values are mean, and the SEM is indicated with a bar (unless it is too small to be shown) (n = 6–12 from at least two different cultures). Note that estimates for 40 mM K^+ in C were obtained only at 7 days *in vitro* (□). No significant change was detectable in the basal formation of [^{3}H]InsP under any of the conditions tested throughout the cultivation period (see also Ref. 11). The standard error of the mean basal values for the data points in the graphs was ≤5%; the range of the mean basal values (in dpm/mg of protein) was 7100–7700 in A, 7400–7700 in B, and 6400–7100 in C.

TABLE 2

Effect of treatment with NMDA, kainate, and AMPA on quisqualate or ACPD-stimulated PPI hydrolysis in cultures grown in 10 mM K^+ medium

NMDA (100 μM), kainate (50 μM), and AMPA (5 μM) were added at 2 days *in vitro*, and the response to quisqualate or ACPD was estimated at 7 days *in vitro*. Values are mean ± standard error (n = 6–15). ANOVA indicated that the agonists (quisqualate or ACPD) significantly increased PPI hydrolysis; in comparison with 10 mM K^+ cultures, treatment with NMDA or kainate, but not with AMPA, significantly increased the metabotropic response.

	[^{3}H]InsP formation		
	Basal	100 μM Quisqualate	300 μM ACPD
	dpm/mg of protein × 10^{-3}		
10 mM K^+	7.5 ± 0.4	21.9 ± 1.2	12.3 ± 0.3
+NMDA	7.8 ± 0.5	62.2 ± 1.2	31.9 ± 1.2
+Kainate	7.2 ± 0.4	40.6 ± 0.7	NT*
+AMPA	7.5 ± 0.5	25.5 ± 1.0	NT

* NT, not tested.

drolysis, it had no significant influence on the CCh-stimulated [^{3}H]InsP formation (Table 3).

In contrast to the effect of NMDA in 10 mM K^+, treatment with 100 μM NMDA resulted in a severe reduction of quisqualate-stimulated PPI hydrolysis in 25 mM K^+ cells during the tested cultivation time (Fig. 1A). This effect was rapidly induced ($t_{½}$ = 20 min), and the potency of NMDA to suppress the metabotropic response in 25 mM K^+ was much lower compared with the effect of NMDA in preventing the developmental decline of the activity in 10 mM K^+ (EC_{50}, approximately 25 versus 4 μM). NMDA suppression of $mGluR_{PI}$ activity was blocked by NMDA receptor antagonists (1 μM MK-801) and counteracted by the simultaneous exposure of the 25 mM K^+ cells to the protein kinase inhibitor H-7 (10 μM) (data not shown).

In contrast to mGluR activity, CCh-stimulated PPI hydro-

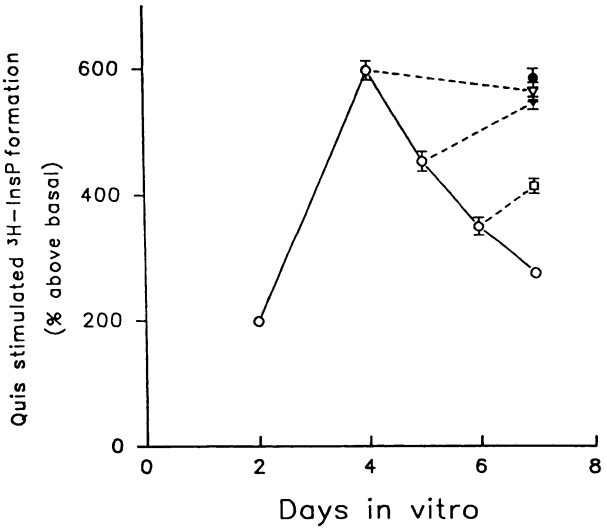

Fig. 2. NMDA treatment of 10 mM K^+ cultures completely prevents the developmental decline of [^{3}H]InsP formation induced by quisqualate (*Quis*) (100 μM) when the treatment was initiated at 2 or 4 days *in vitro*, whereas it restores activity toward the peak value when started at times when the decrease has already taken place. (Values after NMDA supplementation at 5–6 days *in vitro* were significantly different at 7 days *in vitro* from estimates before the NMDA addition [$p < 0.01$ versus 5 days *in vitro* or $p < 0.05$ versus 6 days *in vitro*].) Cultures received 100 μM NMDA at the following days *in vitro*: 2 (●), 4 (▽), 5 (▼), and 6 (□), and [^{3}H]InsP formation evoked by 100 μM quisqualate was estimated at 7 days *in vitro*. In addition, mGluR activity was determined in sister cultures grown in the absence of NMDA at the indicated times. Estimates represent the mean ± standard error (n = 3). Basal [^{3}H]InsP formation was not significantly influenced as a function of either cultivation time or NMDA treatment. The range of the mean basal values was 6100–6600 dpm/mg of protein (the standard error of the mean of the individual mean estimates was ≤5%). The experiment was repeated, using a different culture, with similar results.

TABLE 3

Differences in the effect of NMDA treatment on quisqualate- and carbamylcholine-induced PPI hydrolysis

NMDA (5 or 100 μM) was added on day 2 in culture, and the response to either quisqualate or CCh (each at 100 μM) was tested at 7 days *in vitro*. Values are mean ± standard error (n = 4–6). Percentage of stimulation is reported in brackets. ANOVA showed that in comparison with untreated 10 mM K^+ cultures, exposure to 100 μM NMDA resulted in a significant increase in both the quisqualate- and the CCh-induced PPI hydrolysis (p = 0.05) but that 5 μM NMDA had a significant effect only on $mGluR_{PI}$ activity.

	[^{3}H]InsP formation		
	Basal	Quisqualate	CCh
	dpm/mg of protein × 10^{-3}		
10 mM K^+	6.6 ± 0.4	13.2 ± 0.1 (200%)	13.3 ± 0.5 (201%)
+NMDA (5 μM)	6.1 ± 0.2	20.2 ± 0.3 (331%)	14.6 ± 0.8 (239%)
+NMDA (100 μM)	5.7 ± 0.2	35.2 ± 0.4 (617%)	30.0 ± 0.4 (526%)

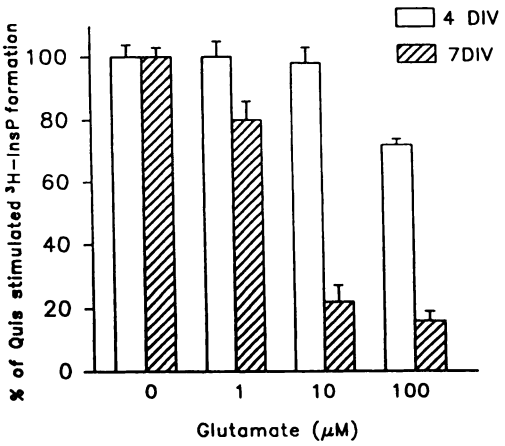

Fig. 3. Desensitization of $mGluR_{PI}$ by glutamate in granule cells grown in 25 mM K^+ for either 4 or 7 days *in vitro*. Cultures were pretreated with Glu for 2 hr in the presence of 1 μM MK-801 and 50 μM DNQX to block ionotropic Glu receptors and, after washing, challenged with 100 μM quisqualate (*Quis*) to stimulate PPI hydrolysis. Estimates are expressed as a percentage of quisqualate-induced [^{3}H]InsP formation in cultures not treated with Glu and represent the mean ± standard error (n = 6–12) from at least two different cultures. The range of the mean basal values was 7200–7500 dpm/mg of protein (the standard error of the mean of individual mean estimates was ≤5%).

lysis was not affected in 25 mM K^+ cultures by NMDA supplementation (Fig. 1C).

Desensitization of $mGluR_{PI}$s in cultures grown under different conditions

At 7 days *in vitro*, a 2-hr preexposure of 25 mM K^+ cultures to glutamate or quisqualate resulted in $mGluR_{PI}$ desensitization, as reflected by the marked decrease in the stimulation of [^{3}H]InsP formation induced by a successive challenge with quisqualate (Fig. 3 and Table 4). Responses to $mGluR_{PI}$ stimulation were similarly reduced after pretreating 25 mM K^+ cells with ACPD (200 μM), and quisqualate-induced desensitization was not influenced by including MK-801 (1 μM) plus DNQX (50 μM) in the pretreatment medium (data not shown). Pretreatment with 1–10 μM glutamate substantially depressed the metabotropic response in 25 mM K^+ cultures at 7 days *in vitro*. In contrast, at 4 days *in vitro*, 10 μM glutamate was completely inactive, and even at 100 μM, the degree of desensitization was relatively low (Fig. 3).

Protein phosphorylation by activated PKC has been implicated in the rapid phase of $mGluR_{PI}$ desensitization in cultured cerebellar granule cells (15, 16, 26). Accordingly, in 25 mM K^+ cultures at 7 days *in vitro*, TPA, a potent activator of PKC, powerfully inhibited quisqualate-stimulated [^{3}H]InsP formation when present during the assay (see Table 5), whereas the desensitizing action of quisqualate was abolished when cultures were preincubated with 100 nM TPA for 24 hr, a condition known to down-regulate PKC activity (27) (Fig. 4). It is noteworthy that TPA (up to 100 nM) failed to reduce quisqualate-induced PPI hydrolysis in 25 mM K^+ cultures at 4 days *in vitro* (Table 5), although the specific binding of [^{3}H]PdBu to intact cells was comparable at 4 and 7 days *in vitro* (data not shown).

In comparison with 25 mM K^+ cells at 7 days *in vitro*, the ability of glutamate or quisqualate to desensitize the $mGluR_{PI}$ was substantially reduced in cultures grown in 10 mM K^+ and apparently abolished in the NMDA-treated 10 mM K^+ cultures (Fig. 5 and Table 4). Although the ability of TPA to suppress quisqualate-stimulated PPI hydrolysis was only somewhat less in 10 mM K^+ than in 25 mM K^+ cells, the desensitizing influence of TPA was virtually lost in the NMDA-treated 10 mM K^+ cultures (Table 5).

Developmental changes in the expression of mGluR1 mRNA in cultures grown under different conditions

Northern blot analysis of total RNA extracted from cultured cerebellar granule cells revealed that under the experimental conditions tested, the mGluR1 mRNA level was highest in cultures grown in 10 mM K^+, with the following rank order: cultures in 10 mM K^+ plus NMDA > 25 mM K^+ > 25 mM K^+ plus NMDA > 40 mM K^+ (Figs. 6 and 7A). This pattern is the opposite of that exhibited by the mRNA for the GluR1 subunit of AMPA receptors (28) and the NMDA receptor-related glutamate-binding protein and mRNA (rank order: 25 mM K^+ > 10 mM K^+ plus NMDA > 10 mM K^+; Ref. 29 and Kumar *et al.*,[1] respectively). In cultures grown in 25 mM K^+, the expression of mGluR1 mRNA was much greater at 4 than at 7 days *in vitro*, which is in agreement with previous results (11). A similar difference was found in cultures grown in 25 mM K^+ plus NMDA or 40 mM K^+, but the decrease with cultivation time in the amount of the mRNA was rather small in the 10 mM K^+ or 10 mM K^+ plus NMDA cells (Fig. 7A).

Discussion

The main findings of this study are the observations that developmental changes in the $mGluR_{PI}$ activity of cerebellar granule cells are subject to regulation by defined environmental factors and that depending on growth conditions, different mechanisms may underlie even apparently similar developmental profiles. Furthermore, these mechanisms involve maturational changes that affect homologous desensitization and interactive effects between processes mediated by ionotropic and metabotropic glutamate receptors.

The environmental factors—either the modulation of the membrane potential by cultivation in media containing different concentrations of K^+ or stimulation of EAA receptors by agonist treatment—were studied because there is reason to believe that they mimic the physiological influence on differentiating granule cells of their afferents, the glutamatergic

[1] Kumar, Michaelis, Resink, and Balázs, unpublished observations.

TABLE 4

Desensitization of mGluR by quisqualate in cerebellar granule cells cultured for 7 days *in vitro* under different grown conditions

Cultures were grown in the media indicated (NMDA, 140 μM, was added at 2 days *in vitro*). At 7 days *in vitro*, 1 μM quisqualate was added for 2 hr, and after its removal by thorough washing of the cultures, cells were challenged with 100 μM quisqualate to elicit PPI hydrolysis. ANOVA indicated that quisqualate pretreatment resulted in a significant decrease in mGluR activity in cultures grown both in 25 mM and 10 mM K^+ but not in the presence of NMDA. Quisqualate pretreatment had no effect on basal [^{3}H]InsP formation. Treatment was for 2 hr.

Culture	[^{3}H]InsP formation					
	25 mM K^+		10 mM K^+		10 mM K^+ + NMDA	
	Basal	Quisqualate	Basal	Quisqualate	Basal	Quisqualate
	dpm/mg protein × 10^{-3}		*dpm/mg protein* × 10^{-3}		*dpm/mg protein* × 10^{-3}	
None	7.4 ± 0.5	23.9 ± 1.0	7.2 ± 0.2	14.4 ± 1.0	7.1 ± 0.5	33.5 ± 1
Quisqualate (1 μM)	7.5 ± 0.4	10.4 ± 0.5	7.2 ± 0.5	11.6 ± 0.5	7.2 ± 1.0	32.4 ± 0.5

TABLE 5

Effect of TPA treatment on quisqualate-stimulated PPI hydrolysis in cultures grown either in 25 mM K^+ for different times or under different growth conditions for 7 days

TPA was added to [^{3}H]inositol-prelabeled granule cells incubated in Krebs-Henseleit buffer 5 min before challenge with 100 μM quisqualate. Values are mean ± standard error (n = 4–8) from at least two different cultures.

Treatment	Quisqualate-stimulated [^{3}H]InsP formation (net increase)			
	25 mM K^+		10 mM K^+:	10 mM K^+ + NMDA:
	4 Days *in vitro*	7 Days *in vitro*	7 Days *in vitro*	7 Days *in vitro*
	dpm/mg protein × 10^{-3}			
None	40.1 ± 0.1	22.4 ± 0.1	22.6 ± 0.2	47.5 ± 0.2
1 nM TPA	39.1 ± 0.4	12.6 ± 0.4[a]	15.1 ± 0.1[a]	48.5 ± 0.3
10 nM TPA	40.5 ± 0.8	5.9 ± 0.3[a]	8.4 ± 0.6[a]	48.0 ± 0.4
100 nM TPA	38.0 ± 0.7	1.9 ± 0.3[a]	1.5 ± 0.3[a]	32.8 ± 0.5[a]

[a] ANOVA indicated significant differences in comparison with control cultures not exposed to TPA.

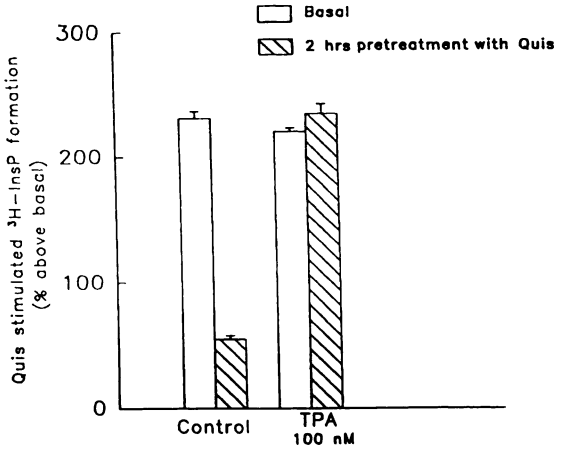

Fig. 4. Pretreatment of granule cells with TPA for 24 hr prevents homologous $mGluR_{PI}$ desensitization. Cultures, grown in 25 mM K^+ for 6 days *in vitro*, were exposed for 24 hr to 100 nM TPA and then pretreated with quisqualate (*Quis*) (100 μM) for 2 hr (to induce desensitization). After removal of the medium, cultures were rechallenged with quisqualate (100 μM). Values are mean ± standard error of six individual determinations. Basal [^{3}H]InsP formation was (in dpm/mg of protein) 7350 ± 50 in control samples and 7580 ± 200 in TPA-treated samples (values after quisqualate pretreatment were 7410 ± 43 and 7430 ± 55, respectively).

mossy fibers (22, 23). Elevated K^+ (≥20 mM) and treatment with EAA receptor agonists are known to have a trophic influence on cultured granule cells (12), and the transduction cascades triggered by $mGluR_{PI}$ activation may exert lasting influences on nerve cells (10). Now we will discuss the influence of chronic exposure to high K^+ and to NMDA on the expression of $mGluR_{PI}$ during development of granule cells *in vitro*.

Mechanisms involved in the developmental decline of $mGluR_{PI}$ activity are different in cells grown in 25 mM K^+ and 10 mM K^+. The comparison of mGluR agonist-induced PPI hydrolysis under the standard "trophic" and the basal "nontrophic" conditions (cultures in 25 and 10 mM K^+ media, respectively) showed that the age courses were similar and were characterized by an early peak (at 4 days *in vitro*) followed by a progressive decline. However, mechanisms underlying the developmental decline under the two conditions were found to be different. In 25 mM K^+ cultures, developmental changes occur that result in an increase of the sensitivity of $mGluR_{PI}$ to agonist-induced desensitization. This was highlighted by the observation that continuous removal of extracellular glutamate by treating cultures with AlAT plus pyruvate prevented the decrease in mGluR agonist-induced PPI hydrolysis after 4 days *in vitro*. Furthermore, the potency of glutamate to desensitize the $mGluR_{PI}$ in 25 mM K^+ cells was approximately 100-fold higher at 7 than at 4 days *in vitro*. The developmental changes affecting homologous desensitization were paralleled by the influence of the PKC activator TPA on $mGluR_{PI}$ activity. TPA powerfully suppressed mGluR-stimulated PPI hydrolysis at 7 days *in vitro* (Table 6), but it was ineffective at 4 days *in vitro*, although both PKC activity (30) and [^{3}H]PdBu binding (pres-

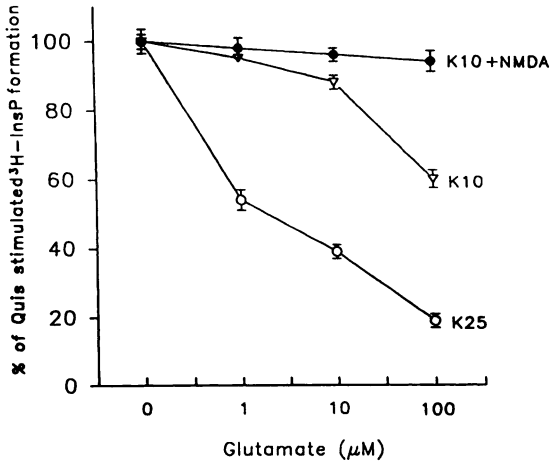

Fig. 5. Desensitization of $mGluR_{PI}$ by glutamate depends on the growth conditions. Granule cells were cultured for 7 days in the different media as indicated. After 2-hr Glu pretreatment (in the presence of 1 μM MK-801 and 50 μM DNQX), cells were challenged with 100 μM quisqualate (*Quis*). Ordinate represents quisqualate-induced [^{3}H]InsP formation as a percentage of estimates in cultures that were grown in the indicated media and were not pretreated with Glu. Values are mean ± standard error (n = 6–12) from at least two different cultures. Basal [^{3}H]InsP formation was (in dpm/mg of protein) 7230 ± 200 in 10 mM K^+, 7370 ± 180 in 10 mM K^+ plus NMDA, and 7340 ± 50 in 25 mM K^+ (pretreatment with Glu at the concentrations tested had no significant effect on the basal values).

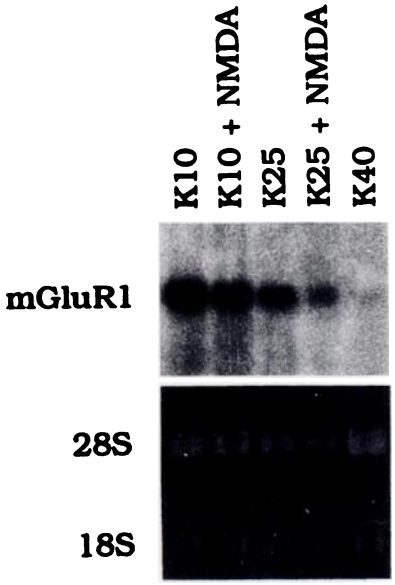

Fig. 6. Northern blot analysis of mRNAs for mGluR1α derived from cultures grown for 7 days in 10 mM or 25 mM K^+ media in the presence or absence of 100 μM NMDA (added at 2 days *in vitro*) and in 40 mM K^+. Each *lane* was loaded with 20 μg of total RNA. Ethidium bromide-stained gel shows the amount of ribosomal RNA (28 S and 18 S) detected in the samples.

ent observations) are detectable at this age and 100 μM glutamate was able to induce a limited desensitization (Table 6). We therefore propose that the development of increased sensitivity to homologous desensitization, which involves PKC-mediated reactions, may play an important role in the decrease with maturation in $mGluR_{PI}$ activity in nerve cells.

mGluR agonist-induced PPI hydrolysis also decreased in 10 mM K^+ cultures after 4 days *in vitro*. However, in contrast to 25 mM K^+ cultures, the decrease was not prevented by AlAT treatment. The reason for the difference in the responses of the 10 and 25 mM K^+ cells to transaminase treatment was found in glutamate potency for $mGluR_{PI}$ desensitization; this was as low in 10 mM K^+ cultures at 7 days *in vitro* as it was in 25 mM K^+ cultures at 4 days *in vitro*. At 7 days *in vitro* mGluR desensitization induced by 100 μM glutamate in 10 mM K^+ cultures was similar to that caused by 1 μM glutamate in 25 mM K^+, implying that in 10 mM K^+ glutamate at the concentration detected in the culture medium (<1 μM) could not have induced $mGluR_{PI}$ desensitization.

In 10 mM K^+ cultures, the decrease in $mGluR_{PI}$ activity during the period of 4 to 7 days *in vitro* is probably related to suboptimal growth conditions. By monitoring various biochemical parameters, we have observed that in comparison with 25 mM K^+ cultures, the maturation of granule cells in 10 mM K^+ usually is retarded (31), and a similar tendency was revealed by analysis of the potency of glutamate for mGluR desensitization in cells grown in 10 and 25 media mM K^+ (see above). Another case in point is the NMDA receptor: the functional expression of these receptors, in terms of NMDA-induced $^{45}Ca^{2+}$ influx, initially increases in culture irrespective of the growth conditions. After 4–5 days *in vitro*, however, in contrast to 25 mM K^+ cultures, receptor activity no longer increases in the 10 mM K^+ cells but instead falls to a lower plateau level (32). Similarly, CCh-induced PPI hydrolysis shows an initial rise in both 10 and 25 mM K^+ cultures, but after 4 days *in vitro*, the muscarinic cholinergic response further increases in 25 mM K^+, whereas it declines in the 10 mM K^+, cells (Fig. 1C). It should be noted that in contrast to 25 mM K^+, cells at 7 days *in vitro*, the correspondence between homologous desensitization and TPA-induced suppression of mGluR activity is less "satisfactory" in the 10 mM K^+ cells, with the potency of TPA being relatively high (only slightly less than in 25 mM K^+), whereas that of glutamate is much lower (about 0.01 of that in 25 mM K^+ cells) (Table 6).

NMDA receptor activation has a profound effect on the functional expression of $mGluR_{PI}$. In an attempt to improve the growth conditions, we treated 10 mM K^+ cultures with NMDA, which is not an mGluR agonist (13) and exerts a trophic influence on granule cells similar to that of elevated K^+ (12, 23, 33–35). However, the effect of NMDA was different

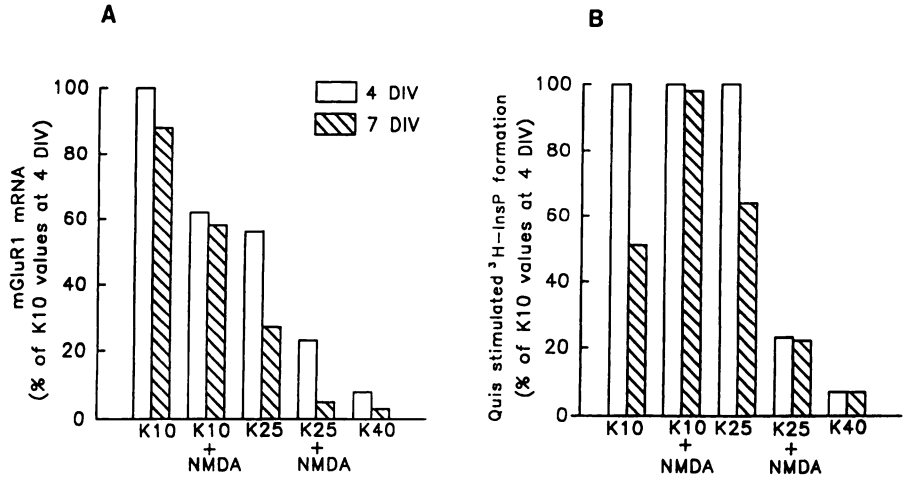

Fig. 7. Comparison of mGluR1α with mRNA levels and mGluR agonist-stimulated PPI hydrolysis in granule cells grown under the indicated conditions for 4 and 7 days. A, mGluR1α mRNA levels; mRNA was extracted, processed, and analyzed as described in Materials and Methods (see also Fig. 6). Data were obtained dividing the densitometric estimates for the mGluR mRNA hybridization band by the corresponding estimate for the ethidium bromide-stained fluorescent ribosomal RNA. Results are expressed as a percentage of the 10 mM K^+ values at 4 days *in vitro*. B, mGluR activity; 100 μM quisqualate (Quis)-induced [^{3}H]InsP formation was determined in cultures grown under the conditions and for the length of time as indicated, and the estimates were expressed as a percentage of the value in 10 mM K^+ cells at 4 days *in vitro*. Data are derived from a minimum of two cultures (the standard error of the mean of the absolute values was <10% of the mean under all the conditions represented).

TABLE 6

Influence of growth conditions on homologous desensitization and TPA suppression of mGluR agonist-induced PPI hydrolysis[a]

	4 Days *in vitro*		7 Days *in vitro*	
	Glutamate	TPA	Glutamate	TPA
25 mM K^+	+	0	+++	+++
10 mM K^+			+	+++
+NMDA			0	+

[a] +++, Reduction in $mGluR_{PI}$ activity by pretreatment with glutamate (or quisqualate) or by exposure to TPA is pronounced: suppression approximately 80% at high glutamate (10–100 μM) or TPA (100 nM). +, Slight, <40% at the highest concentration of glutamate or TPA tested). 0, not significant.

than that of elevating $[K^+]_e$ to 25 mM. NMDA counteracted the developmental decline of mGluR agonist-induced PPI hydrolysis in 10 mM K^+ cultures, and significantly, the effect involved the prevention of homologous desensitization. NMDA treatment also resulted in a pronounced decrease (although not a complete blockade) of the effect of TPA in suppressing $mGluR_{PI}$ activity (Table 6). Pharmacological studies have shown that the effect of NMDA is mediated specifically through NMDA receptors. The potency of NMDA to prevent the developmental decline of mGluR activity was about 10-fold higher than the effect of NMDA in promoting cell survival in 10 mM K^+ cultures (EC_{50}, approximately 4 and 38 μM; Ref. 23). Furthermore, NMDA was much more potent in affecting $mGluR_{PI}$ than in influencing muscarinic cholinergic receptors, whose activity was elevated only at NMDA concentrations that exerted a generalized trophic influence. The effect of NMDA was manifest after a delay of about 24 hr and required new protein synthesis. These characteristics suggest that the effect is mediated through inducible factor(s), whose nature remains to be established.

The effect of NMDA was remarkably different in 25 mM K^+ from that in the "low" K^+ cultures. In 25 mM K^+ cells, the glutamatergic metabotropic response was depressed by NMDA. In comparison with 10 mM K^+ cultures, in 25 mM K^+ the effect of NMDA not only occurred in the opposite direction (inhibition rather than maintenance or restoration of high activity) but also occurred more rapidly ($t_{½}$, 20 min versus many hours in 10 mM K^+), and the potency of NMDA was much lower (EC_{50}, 25 versus 4 μM). It should be noted that the effect of NMDA on the functional expression of NMDA receptors is also dependent on the external K^+ concentration. In 10 mM K^+, NMDA supports a progressive increase in receptor activity (32), whereas in 25 mM K^+, NMDA treatment results in a massive decrease in NMDA receptor function, as assessed by tests of NMDA-induced $^{45}Ca^{2+}$ influx and toxicity.[2] H-7 counteracted the NMDA-induced suppression of $mGluR_{PI}$ activity in 25 mM K^+ cells. This observation suggests that NMDA receptor "desensitization" may involve the phosphorylation of the receptor. Courtney and Nicholls (36) have reported a decrease in NMDA receptor function in terms of $[Ca^{2+}]_i$ elevation after PKC activation with TPA. It therefore appears that NMDA receptor-mediated processes are critically involved in the maintenance of high $mGluR_{PI}$ activity, probably by counteracting $mGluR_{PI}$ desensitization.

Mechanisms underlying the severe depression of mGluR agonist-induced PPI hydrolysis in 40 mM K^+ cells. K^+ concentrations in the range of 20–40 mM have similar, positive effects on cell survival (22), whereas 40 mM K^+ appears to be optimal for the promotion of granule cell maturation, as assessed by monitoring glutaminase activity (37). In contrast, in this study we observed that mGluR function is severely depressed in 40 mM K^+ cultures. Compared with 25 mM K^+, glutamate was elevated in the media of 40 mM K^+ cultures from 2 to 4 μM. Assuming that the desensitizing potency of glutamate in 40 mM K^+ cultures is similar to that in 25 mM K^+ cultures (EC_{50}, approximately 4 μM), such an increase in $[Glu]_e$ may have contributed to a more pronounced receptor desensitization. We observed that in 40 mM K^+ cultures at 7 days *in vitro*, quisqualate-induced [^{3}H]InsP formation was increased from 18 to 170% over the basal level by transaminase treatment initiated at 4 days *in vitro*. However, in comparison with 25 mM K^+ cultures that were treated similarly, quisqualate-induced PPI hydrolysis was only about 40% in the $[Glu]_e$-depleted 40 mM K^+ cultures at 7 days *in vitro*, indicating that under these conditions the removal of medium glutamate can only prevent, in part, the suppression of $mGluR_{PI}$ activity. It should be noted, however, that mGluR1 mRNA levels are very low in cells grown in the 40 mM K^+ medium (Fig. 7A), and these may become rate limiting for the overall glutamatergic metabotropic response.

Comparison of $mGluR_{PI}$ activity and mRNA levels. Our observations permitted the comparison of the effect of K^+-induced membrane depolarization and of NMDA treatment on $mGluR_{PI}$ function and on $mGluR_{PI}$ mRNA levels. The findings confirmed recent observations (16, 17) that $[K^+]_e$ elevation results in a depression of mGluR1 mRNA levels. In addition, we observed that the degree of the depression is dependent on the concentration of K^+. Both mGluR activity and mRNA levels attained the highest estimates in 10 mM K^+ cultures at 4 days *in vitro*. By 7 days *in vitro*, mGluR activity decreased by 50% in the 10 mM K^+ cultures, whereas mRNA levels were reduced by only approximately 10% (Fig. 7). Although in comparison with cultures grown in 10 mM K^+, quisqualate-induced PPI hydrolysis in 25 mM K^+ was similar at 4 days *in vitro* and even somewhat higher at 7 days *in vitro*, mRNA levels were only one-half and one-third, respectively.

Discrepancies were also evident in the effects of NMDA treatment on the mGluR-induced PPI hydrolysis and mGluR1 mRNA levels (Fig. 7). In both 10 mM K^+ and 25 mM K^+ media, NMDA resulted in a decrease in mRNA levels, although activity was elevated in 10 mM K^+ cultures (after 4 days in *in vitro*) and decreased in 25 mM K^+ cultures (throughout cultivation). Both mRNA levels and $mGluR_{PI}$ activities remained relatively constant during the period of 4–7 days *in vitro* in the NMDA-treated 10 mM K^+ cultures, whereas mRNA levels, but not $mGluR_{PI}$ activities, decreased substantially on NMDA exposure of 25 mM K^+ cells by 7 days *in vitro*. These observations imply that the regulation of the functional expression of $mGluR_{PI}$ involves factors in addition to the receptor mRNA levels.

Interactive effects involving NMDA receptors, $mGluR_{PI}$, and synaptic plasticity. It is noteworthy that NMDA treatment of 10 mM K^+ cultures not only prevented the age-dependent reduction of but also induced an increase in $mGluR_{PI}$ activity when NMDA was added after the peak of the metabotropic response had already been reached, *i.e.*, at 5–6 days *in vitro*. This suggests that the activation of NMDA receptors under appropriate conditions can in part restore the functional expression of $mGluR_{PI}$ in adult life. Such a mechanism may have important implications in connection with

[2] Resink *et al.*, in preparation.

synaptic plasticity and may contribute toward a better understanding of the significance of the observed increase in $mGluR_{PI}$ activity during electrical "kindling" (4, 5), LTP (6), acquisition of spatial learning (7), and development of ischemic damage (8, 9), all these conditions being triggered by an initial activation of NMDA receptors (38).

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