Transient and Persistent Phosphorylation of AMPA-Type Glutamate Receptor Subunits in Cerebellar Purkinje Cells

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Summary

We generated a polyclonal antibody, 12P3, specifically recognizing rat AMPA-type glutamate receptor (GluR) subunits phosphorylated at Ser-696 of GluR2 or at the homologous sites in GluR1, GluR3, and GluR4. Using 12P3, we demonstrate that a brief exposure of a rat cerebellar slice to AMPA leads to transient phosphorylation of the GluR subunits in Purkinje cell dendrites. Persistent phosphorylation over 30 min was obtained when exposure to AMPA was preceded by a 15 min perfusion of the slice with 8-bromo-cGMP, dibutyryl-cGMP, or calyculin A but not phorbol 12,13-diacetate. These results indicate that Ser-696 of GluR2, or the corresponding sites in other AMPA receptor subunits, is a specific site at which phosphorylation takes place when AMPA-type GluRs are activated by agonists, especially under the influence of certain second messenger activities.

Introduction

Ionotropic glutamate receptor (GluR) phosphorylation is postulated to have a role in receptor desensitization and long-term synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD) (reviewed by Raymond et al., 1993a; Soderling et al., 1993; Roche et al., 1994). With recent evidence indicating that both the N-methyl-D-aspartate (NMDA) receptors (Tingley et al., 1993; Tingley and Huganir, 1994, Soc. Neurosci., abstract) and the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainate receptors (Moss et al., 1993; McGlade-McCulloh et al., 1993; Raymond et al., 1993b; Tan et al., 1994; Nakazawa et al., 1994, Soc. Neurosci., abstract), both of which comprise ionotropic GluRs, are directly phosphorylated, the phosphorylation sites of each GluR subunit have been revealed, and some of their roles have been investigated. For example, substitution of the phosphorylation sites by site-directed mutagenesis (i.e., Ser-684 of GluR6 [Wang et al., 1993; Raymond et al., 1993b] or Ser-627 of GluR1 [Yakel et al., 1995]) has eliminated agonist-induced current potentiation, suggesting the involvement of receptor phosphorylation in synaptic transmission. Moreover, a variety of evidence from hippocampal LTP experiments has suggested that GluR phosphorylation is involved in synaptic plasticity (reviewed by

Meffert et al., 1991; Bliss and Collingridge, 1993; Soderling et al., 1993).

In the cerebellum, LTD at the parallel fiber–Purkinje cell synapses is evoked by conjunctive stimulation of climbing and parallel fibers, the two excitatory inputs to the Purkinje cells, and is thought to be due to a persistent decrease in postsynaptic AMPA receptor sensitivity (reviewed by Ito, 1989, 1991; Crepel and Audinat, 1991; Linden and Connor, 1991; Linden, 1994). This long-lasting reduction of AMPA receptor sensitivity, designated long-term desensitization, is also elicited by application of a membrane-permeable analog of cGMP, 8-bromo-cGMP (8-Br-cGMP), followed by AMPA stimulation using grease-gap (wedge) recording (Ito and Karachot, 1989), thus indicating common intracellular metabolic pathways with synaptically induced LTD (Ito and Karachot, 1990, 1992). Furthermore, a substantial body of evidence from both synaptic LTD and long-term desensitization experiments using activators and inhibitors of protein kinases and phosphatases has suggested that AMPA receptor phosphorylation is involved in cerebellar LTD (Ito, 1991; Linden and Connor, 1991; Ito and Karachot, 1992).

To examine whether AMPA receptors in Purkinje cells could be phosphorylated with the induction of cerebellar LTD, we produced a polyclonal antibody against a synthetic peptide, 690-VARVRKS(PO₄)KGKYA-701, of rat GluR2 (same as GluRB) phosphorylated at Ser-696, and named it 12P3 (Nakazawa et al., submitted). Using this antibody, we show here that 12P3 specifically recognizes the phosphorylated AMPA-type GluR subunits and that an increase in phosphorylation is also detected in cerebellar homogenates made within 5 min after AMPA application to the slices. Furthermore, we demonstrate an AMPA-induced transient phosphorylation of AMPA receptors in Purkinje cell dendrites and its prolongation in response to stimuli that cause long-term desensitization.

Results

Antibodies were generated in a rabbit immunized with Ser-phosphorylated peptide-12 (peptide-12P), which corresponds to amino acids 690–701 of rat GluR2 (690-VARVRKS(PO₄)KGKYA-701; underlined in Figure 1). After extensive purification by peptide-affinity chromatography, antibody 12P3 was obtained, which reacted with peptide-12P but not with nonphosphorylated peptide-12 on enzyme-linked immunosorbent assay (ELISA) plates (Nakazawa et al., submitted).

Antibody 12P3 Specifically Recognizes the Phosphorylated AMPA Receptor Subunits
In cerebellar slice homogenate immunoblots, 12P3 recognized a major band corresponding to the predicted molecular masses of AMPA receptor subunits (~ 105 kDa; Figure 2A, arrow). This band was abolished by preadsorption of 12P3 with peptide-12P (lanes 3 and 4). Treatment of
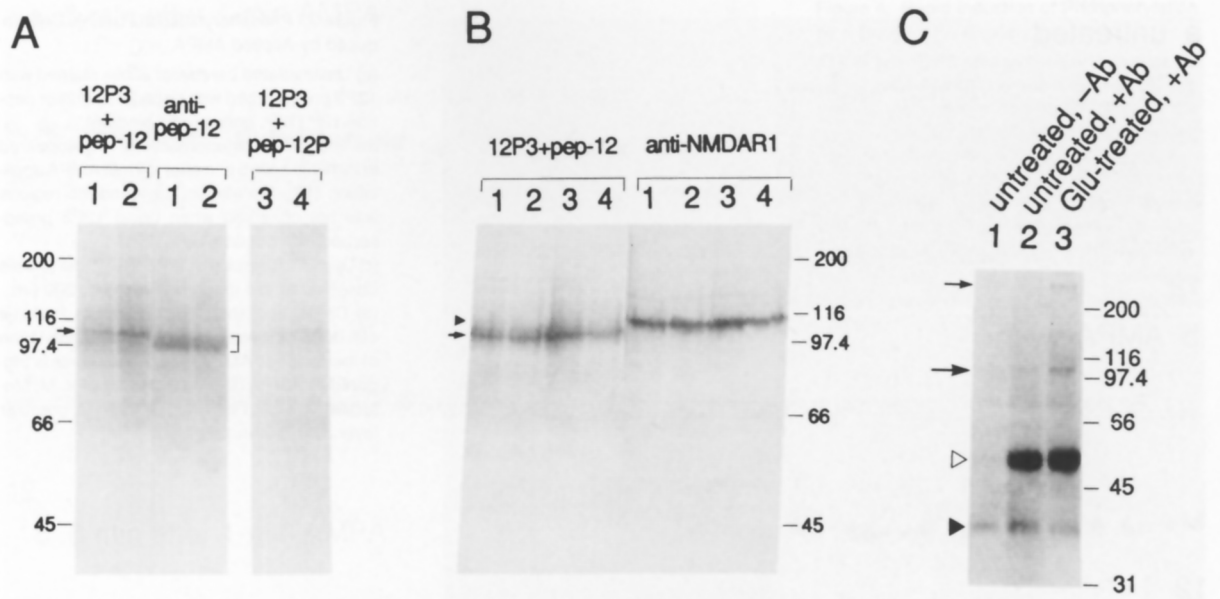
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┌────TM3────┐                    A      DS           YE  T  R    678   GluR4
│           │                    A      EA           FE  T  K    673   GluR1
│           │              +     A      DS      *    YE  S  K    681   GluR3
IVGGVWWFFTLIIISSYTANLAAFLTVERMVSPIESAEDLSKQTEIAYGTLDSGSTKEFFRRSKIAVFDKMWTYMRSA   677   GluR2


   TR  AE  VA      F F    (T)                 V      S Q ┌─A Flop ──┐  756   GluR4
   VR  EE  MI  (T) Y Y                         I      A R │ P        │  751   GluR1
   TK  AD  VA  *  F F                         V      A Q │ A        │  759   GluR3
EPSVFVRTTAEGVARVRKSKGKYAYLLESTMNEYIEQRKPCDTMKVGGNLDSKGYGIATPKGSSLQNAVNIAVLKLNE   755   GluR2


┌──────────────┐ S    D  ┌──TM4─ V      L ┐   A A   LTFSEAT   834   GluR4
│              │ T    D  │       I      L │   S S   GFCLIPQ   829   GluR1
│              │ S    D  │       V      M │   A S   LTKNTQN   837   GluR3
QGLLDKLKNKWWYDKGECGSGGGDSKEKTSALSLSNVAGVFYILVGGLGLAMLVALIEFCYKSRAEAKRMKVAKNPQN   833   GluR2


              RNKARLSITGSVGENGRVLTPDCPKAVHTGTAIRQSSGLAVIASDLP             881   GluR4
              QSINEAIRTSTLPRNSGAGASGGGGSGENGRVVSQDFPKSMQSIPCMSHSSGMPLGATGL 889   GluR1
              FKPAPATNTQNYATYREGYNVYGTESVKI                               866   GluR3
              LNPSSSQNSQNFATYKEGYNVYGIESVKI                               862   GluR2
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Figure 1. Topological Representation of Possible Phosphorylation Sites of Rat AMPA Receptor Subunits

The polypeptide sequence for GluR2, including transmembrane domain 3 (TM3), a flop cassette, and the C-terminal are shown juxtaposed with the sequences for GluR1, GluR3, and GluR4 to identify the peptides that are different from those in GluR2. Underlined amino acids indicate the epitope recognized by 12P3 when the central Ser residue is phosphorylated. Ser-662 and Ser-696 of GluR2 and the corresponding sites of GluR1/3/4 (identified by asterisks) are possible phosphorylation sites of AMPA-type GluR subunits (Nakazawa et al., submitted). Ser-627 of GluR1 as a Ca^{2+}/calmodulin-dependent protein kinase II phosphorylation site (Yakel et al., 1995) is indicated by a plus sign. Sequences, presented in single-letter code and numbered on the right in the order of GluR4, GluR1, GluR3, and GluR2, were predicted from the complete nucleotide sequences listed in the GenBank-EMBL database under accession numbers M85037 (GluR4), X17184 (GluR1), M85036 (GluR3), and M85035 (GluR2) (see Hollmann et al., 1989; Boulter et al., 1990; Bettler et al., 1990). Thr-692 of GluRA and Thr-713 of GluRD (Keinänen et al., 1990) are also shown in parentheses. The C-terminal 36 amino acids of GluR4c divergent from Leu-828 of GluR4, 25 of which share homology with the corresponding C-terminal amino acids present in GluR2, are not shown (Gallo et al., 1992).

the slices with AMPA (10 μM; 4 min) led to an increase in the amount of phosphorylated AMPA receptor subunits 5 min after slice treatment (left lane 2). Since the 12P3-recognized band was sharper than that reacted with anti-peptide-12 (middle lanes 1 and 2), not all the AMPA receptor subunits were phosphorylated in the cerebellar slice after AMPA treatment. Since the peptide-12 sequence from GluR2 is highly homologous to the corresponding sequences of GluR1/3/4, anti-peptide-12 probably recognized all the AMPA receptor subunits regardless of the Ser-phosphorylation state. Furthermore, the broad anti-peptide-12 band seemed to result not only from differences in molecular mass among the four major AMPA receptor subunit core proteins present in the cerebellum (99.8, 96.4, 98.0, and 101.0 kDa for rat GluR1–GluR4, respectively; see Hollmann et al., 1994) but also from a difference in N-linked glycosylation (Rogers et al., 1991; Hattori et al., 1994; Hollmann et al., 1994; Bennett and Dingledine, 1995). The 12P3-immunoreactive band was located nearly at the upper border of the broad anti-peptide-12-immunoreactive band. This may suggest that the phosphorylated AMPA receptor subunits recognized by 12P3 are mature glycoproteins integrated into the plasma membrane, or alternatively, it may be caused by an upward mobility shift of the phosphorylated proteins, probably as a consequence of the conformational change in the attached phosphate groups (as exemplified by the τ protein; Butler and Shelanski, 1986; Arioka et al., 1993).

Moreover, as shown in blots of the membrane fraction of cerebellar slice homogenates immunoprecipitated with the C-terminal antibody of rat GluR2/3/4c (Figure 2C), a 105 kDa 12P3-immunoreactive band, which was almost not visible in untreated homogenates (lane 2), was intensified 5 min after the slices were stimulated with 0.5 mM glutamate (lane 3). An additional higher molecular weight component near the origin of the gel was also phosphorylated (lane 3, small arrow). The AMPA receptor complex with this high molecular weight form retained even after electrophoresis in the presence of SDS has been reported to be immunoprecipitated with the C-terminal antibody of rat GluR2/3/4c (Wenthold et al., 1992; Puchalski et al., 1994), implying that AMPA receptors are phosphorylated as a hetero-oligomer. These results suggested that 12P3 specifically recognizes the phosphorylated AMPA receptors and that the Ser-696 residue in GluR2, or corresponding sites in other AMPA receptor subunits, is efficiently phosphorylated within 5 min following AMPA or glutamate stimulation.

When the cerebellar slices were treated with 0.5 mM 8-Br-cGMP for 15 min prior to AMPA, the signal intensity of 12P3 immunoreactivity (12P3-IR) on the blot was still slightly augmented 30 min after the cessation of drug application, as compared with that of untreated slice homogenates (Figure 2B). In contrast, the degree of 12P3-IR 30 min after AMPA application alone, or after AMPA followed by 8-Br-cGMP, was returned to that of untreated slice ho-

Figure 2. Immunochemical Identification of AMPA Receptor Subunit Phosphorylation in Rat Cerebellar Slice Homogenates

Parasagittal cerebellar slices (400 μm thick) incubated in various experimental protocols were homogenized, fractionated by 7.5% SDS–PAGE, and transferred to PVDF membrane. Positions of molecular mass markers (Bio-Rad) in kilodaltons are indicated on each side.

(A) A blot of slice homogenates (100 μg each) without any treatment (lane 1) or 5 min after a 4 min bath application of AMPA (lane 2) following a 40 min preincubation with 1 μM BAPTA-AM and 0.5 μM TTX was first stained with 12P3 preadsorbed with peptide-12 (left). The same blot (middle) was stripped of bound antibody and then reprobed with anti-peptide-12 antibody. Antibody 12P3 specifically recognizes a 105 kDa band (arrow), which is included in the broad band revealed by anti-peptide-12 (bracket). In contrast, the 12P3-immunoreactive band of another blot (right) is largely eliminated following preadsorption of 12P3 with peptide-12P (100 ng/ml; lanes 3 [untreated] and 4 [5 min AMPA-stimulated]).

(B) Slices were treated with the following: lane 1, untreated; lane 2, AMPA (10 μM; 4 min); lane 3, 8-Br-cGMP (0.5 mM; 15 min) followed by AMPA (10 μM; 4 min); lane 4, AMPA (10 μM; 4 min) followed by 8-Br-cGMP (0.5 mM; 15 min). All slices were homogenized 30 min after cessation of the drug application. The blot of slice homogenates (100 μg each) was stained with 12P3 antibody preadsorbed with peptide-12 (left; arrow). A 105 kDa band recognized by 12P3 was enhanced only in lane 3. After deprobing, the same blot was restained with an antibody directed against the C-terminal of NMDAR1 (right; arrowhead), indicating that the loaded protein amount is the same.

(C) AMPA receptor subunits, GluR2/3/4c, were immunoprecipitated using their C-terminal antibodies (+Ab) from crude membrane fractions (40 μg each) of cerebellar slice homogenates without any treatment (lane 2) or 5 min after a 4 min bath application of glutamate (lane 3) and examined by immunoblot analysis with 12P3 preadsorbed with peptide-12. Immunoprecipitants from untreated membrane fraction without adding the C-terminal antibody (–Ab) were also loaded in lane 1. The receptor phosphorylation detected at M$_r$ 105 kDa (large arrow) was augmented following glutamate treatment. The high molecular bands at M$_r$ 220–230 kDa (small arrow) in lanes 2 and 3 are unidentified, but they might be incompletely reduced complexes of AMPA receptors (Wenthold et al., 1992; Puchalski et al., 1994). C-terminal antibody to GluR2/3/4c (open arrowhead) and a putative recombinant protein G (closed arrowhead) were visualized with BCIP/NBT by binding alkaline phosphatase–conjugated anti-rabbit (secondary) antibody. The protein G, probably leaked from protein G–Sepharose 4FF, was also detected in the boiled fraction of protein G–Sepharose 4FF alone (data not shown).

mogenates. The protein amount loaded on each lane was the same among the four protocols, which was confirmed by NMDAR1-IR. These results imply that 8-Br-cGMP pretreatment prior to AMPA treatment has a tendency to cause a sustained AMPA receptor phosphorylation.

To confirm the results of immunoblots as described above and determine the cell type carrying 12P3-IR, 12P3 immunocytochemical methods were applied to the cerebellar slices. Since some 12P3-IR of nonphosphorylated peptides was observed in ELISA analyses, all immunocytological examinations of the slices were performed using 12P3 preloaded with peptide-12, which was compared with the control IR done simultaneously using 12P3 preadsorbed with peptide-12P.

AMPA-Induced Phosphorylation of AMPA Receptor Subunits in Purkinje Cell Dendrites

Sections (20 μm) from 400 μm thick cerebellar slices fixed after a 40 min incubation in Krebs' solution containing 0.5 μM tetrodotoxin (TTX) and 1 μM BAPTA-AM showed slight 12P3-IR in the somata of Purkinje cells and no labeling in the dendrites at the light microscopic level (Figure 3a). However, 12P3 preadsorbed with peptide-12P showed no IR in other sections from the same slices, indicating some basal presence of phosphorylated AMPA receptor subunits in Purkinje cell somata.

In sections prepared from slices 5 min after stimulation with 10 μM AMPA for 4 min, 12P3-IR was found in Purkinje cell dendrites throughout the molecular layer and in the cytoplasm of their cell bodies (Figure 3b). The intensity of this IR varied among the different folia; in some, only the somata were occasionally stained, and not all dendrites showed IR following bath application of AMPA. However, the overall pattern of IR 5 min after stimulation (as exemplified by Figure 3c) was the same. This staining was abolished from the dendrites and largely eliminated from the somata in sections treated with 12P3 preadsorbed with peptide-12P. The application of the competitive non-
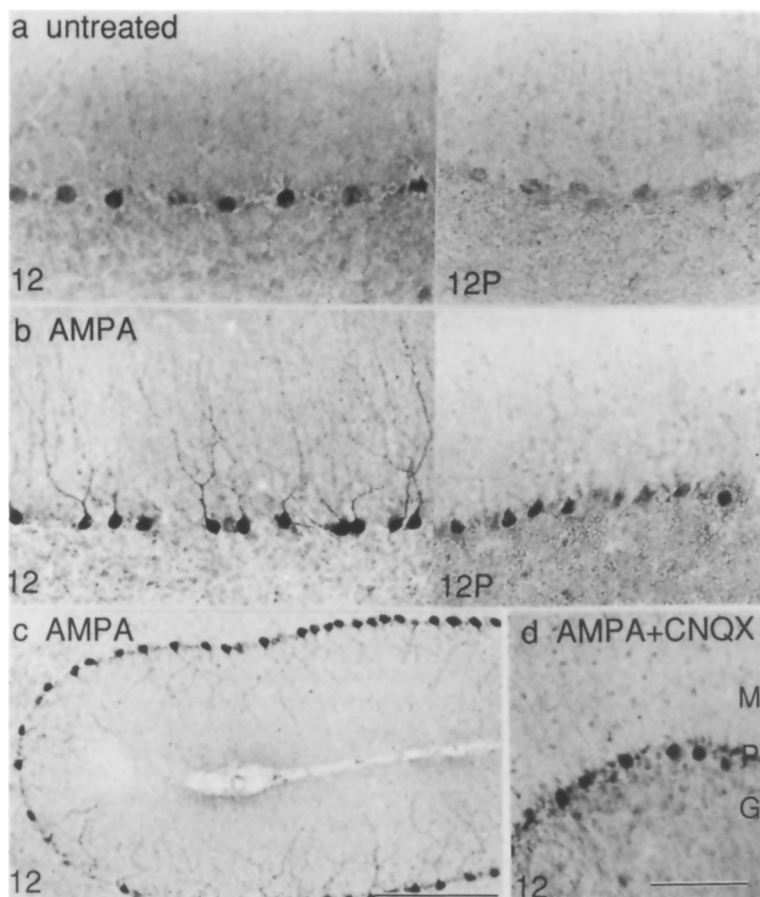
Figure 3. Phosphorylation in Purkinje Cells Induced by Applied AMPA

(a) Unstimulated cerebellar slices stained with 12P3 preadsorbed with peptide-12 (12) or peptide-12P (12P; nonspecific binding).

(b) The 12P3-immunoreactivity (IR) appeared in Purkinje cells 5 min after a 4 min AMPA application (12). Staining in the dendritic regions was not observed when using 12P3 preadsorbed with peptide-12P (12P).

(c) Overall expression of 12P3-IR in Purkinje cells observed at low magnification. Bar, 200 μm.

(d) CNQX abolished the 12P3-IR in Purkinje cell dendrites, indicating that phosphorylation of the dendritic AMPA receptor subunits is triggered by AMPA binding to its receptor. M, Molecular layer; P, Purkinje cell layer; G, granular layer. Bar, 100 μm.

NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) 15 min before and during AMPA application also diminished the labeling in Purkinje cells (Figure 3d). Within 2 min after the cessation of AMPA application for 4 min, 12P3-IR was already observed on the somata and primary dendrites as a gradient from the somata to the tips of the dendrites, indicating the occurrence of an AMPA-induced rapid phosphorylation event (Figure 4b). A 1 min application of 10 μM AMPA was also effective in inducing 12P3-IR (Figure 4c). This IR was in the soma and middle parts of the dendrites, but not as strong in the primary dendrites, which was slightly different from the 12P3-IR observed in Figure 4b.

Kinases Involving Agonist-Induced AMPA Receptor Phosphorylation

Staurosporine (Tamaoki, 1991) and/or K252a (Hashimoto et al., 1991), both potent but relatively nonspecific protein kinase inhibitors, were added to the slice incubation media to test the 12P3-IR of the Purkinje cells. The simultaneous treatment by staurosporine (1 μM) and K252a (0.1 μM) clearly suppressed AMPA-induced 12P3-IR of the Purkinje cell dendrites, but a slight staining of the somata could still be observed (Figure 5c). However, treatment with either staurosporine (1 μM) or K252a (0.1 μM) showed a partially reduced 12P3-IR (Figures 5a and 5b). A high dose (1 μM) of K252a completely abolished the 12P3-IR from the den-

drites (data not shown). A selective protein kinase C (PKC) inhibitor, calphostin C, which competes with phorbol esters at the regulatory site of PKC (applied at 0.5 μM; Tamaoki, 1991; IC_{50} for PKC = 0.05 μM), and a selective protein kinase G (PKG) inhibitor, KT5823 (applied at 0.5 μM; Nakanishi, 1989; K_i for PKG = 0.2 μM), failed to suppress the 12P3-IR (Figures 5d, 5e, and 5f). Even at a large dose (2 μM), KT5823 showed virtually no effect on the 12P3-IR (data not shown). At 5 μM, the membrane-permeable Ca^{2+} chelator, BAPTA-AM, also did not inhibit the 12P3-IR, whereas 10 μM BAPTA-AM treatment slightly diminished the 12P3-IR (data not shown). These results imply that the kinases phosphorylating native GluR subunits in Purkinje cells following applied AMPA are not only PKC or PKG.

Persistent Phosphorylation of Purkinje Cell AMPA Receptors Following Application of Chemical Stimuli Causing Long-Term Desensitization

Application of 8-Br-cGMP for 15 min followed by AMPA for 4 min in the presence of 0.5 μM TTX and 1 μM BAPTA-AM, which causes long-term desensitization (Ito and Karachot, 1992), resulted in about the same degree of 12P3-IR and the same number of stained Purkinje cell dendrites as at 5 min after AMPA application alone. However, 12P3-IR was also observed at 30 min after the combined protocol (Figures 6a and 6b). While the staining intensity was dimin-
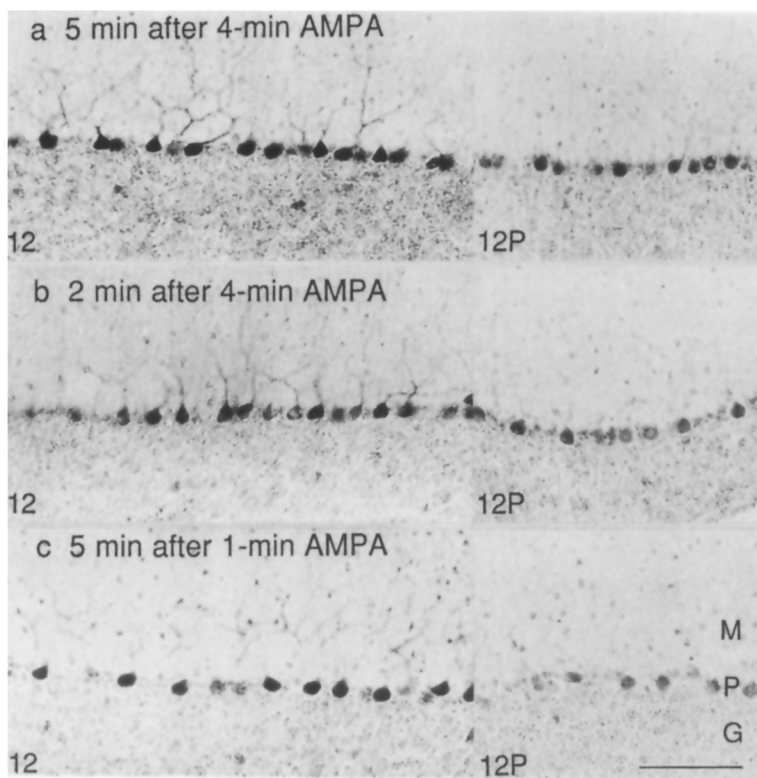
Figure 4. Rapid Induction of Phosphorylation by a Brief AMPA Exposure

The IR with 12P3 preadsorbed with peptide-12 (12) or peptide-12P (12P) as indicated. (a), (b), and (c) are sections from three slices of the same cerebellar vermis, treated as indicated in each. Note that 5 min after AMPA application (a), IR is more marked than at 2 min (b), indicating that phosphorylation is not immediate. However, as seen in (c), presence of AMPA for 1 min is sufficient to initiate phosphorylation. Note in (c) that the marking is less pronounced in the proximal dendrites than in (a) or (b). Bar, 100 µm.
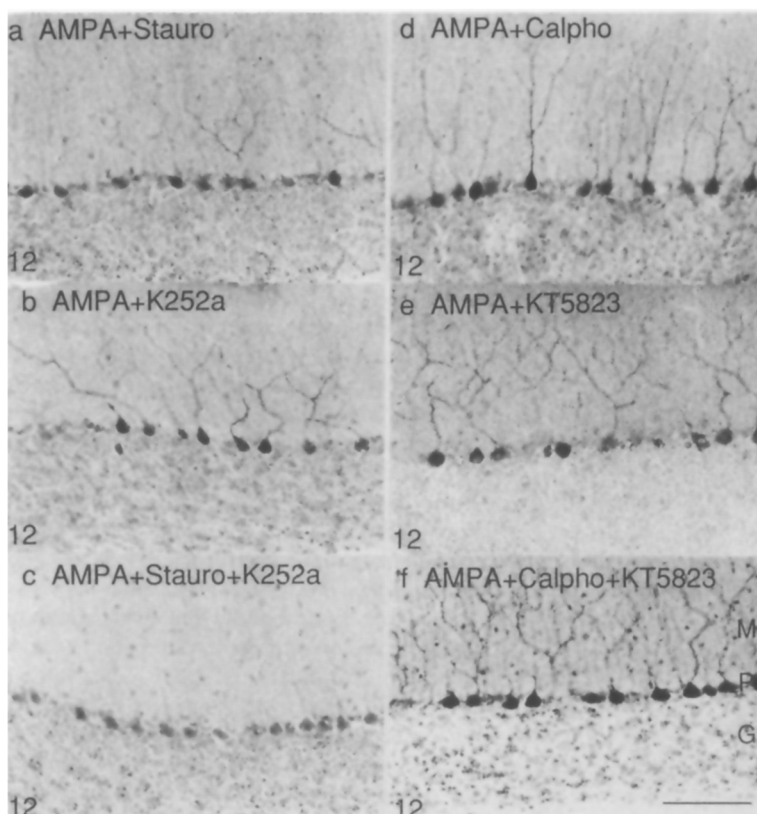
Figure 5. Inhibition of 12P3-IR by Kinase Inhibitors

Protein kinase inhibitors dissolved in dimethylsulfoxide were applied in the incubation medium 40 min before the 4 min AMPA application. Slices were transferred to AMPA-free medium after AMPA application for 5 min and then fixed.

(Left panels) Nonspecific protein kinase inhibitors: 1 µM staurosporine (a), 0.1 µM K252a (b), and simultaneous application of staurosporine and K252a (c).

(Right panels) Effects of selective inhibitors: PKC inhibition with 0.5 µM calphostin C (Calpho; d); PKG inhibition with 0.5 µM KT5823 (KT5823; e); simultaneous treatment of calphostin C and KT5823 (f).

These results indicate that the 12P3-IR-inducing event is a very dominant, kinase-dependent phosphorylation (a–c), and it is not mediated by only PKC and PKG (d–f). Bar, 100 µm.
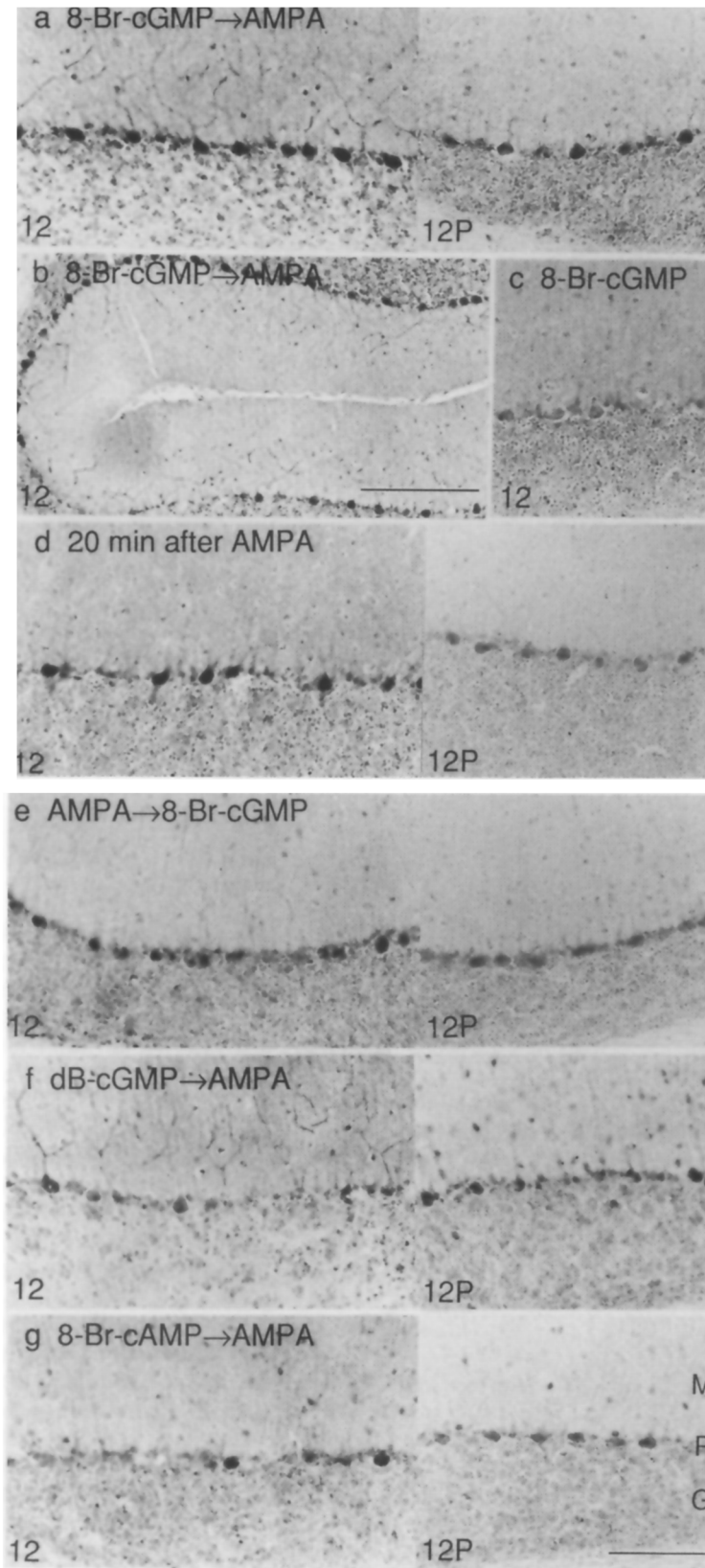
Figure 6. Persistent Phosphorylation by Chemical Stimulation Producing Long-Term Desensitization

Parasagittal cerebellar slices (400 μm thick) were incubated with 8-Br-cGMP, dB-cGMP, or 8-Br-cAMP for 15 min, then activated by a 4 min exposure to AMPA (10 μM). Slices were fixed 30 min after the treatment, except for that in (d), which was fixed after 20 min. Sections reacted with 12P3 preadsorbed with peptide-12 (12) or peptide-12P (12P) as indicated in adjacent panels.

(a and b) 8-Br-cGMP before AMPA; (c) 8-Br-cGMP only; (d) AMPA only; (e) AMPA before 8-Br-cGMP; (f) dB-cGMP before AMPA; (g) 8-Br-cAMP before AMPA. Results obtained with protocols producing long-term desensitization (a, b, and f) indicate that cGMP elevation in Purkinje cell dendrites prior to AMPA binding to the receptor leads to persistent AMPA receptor phosphorylation. Bars, 200 μm (b), 100 μm (g).
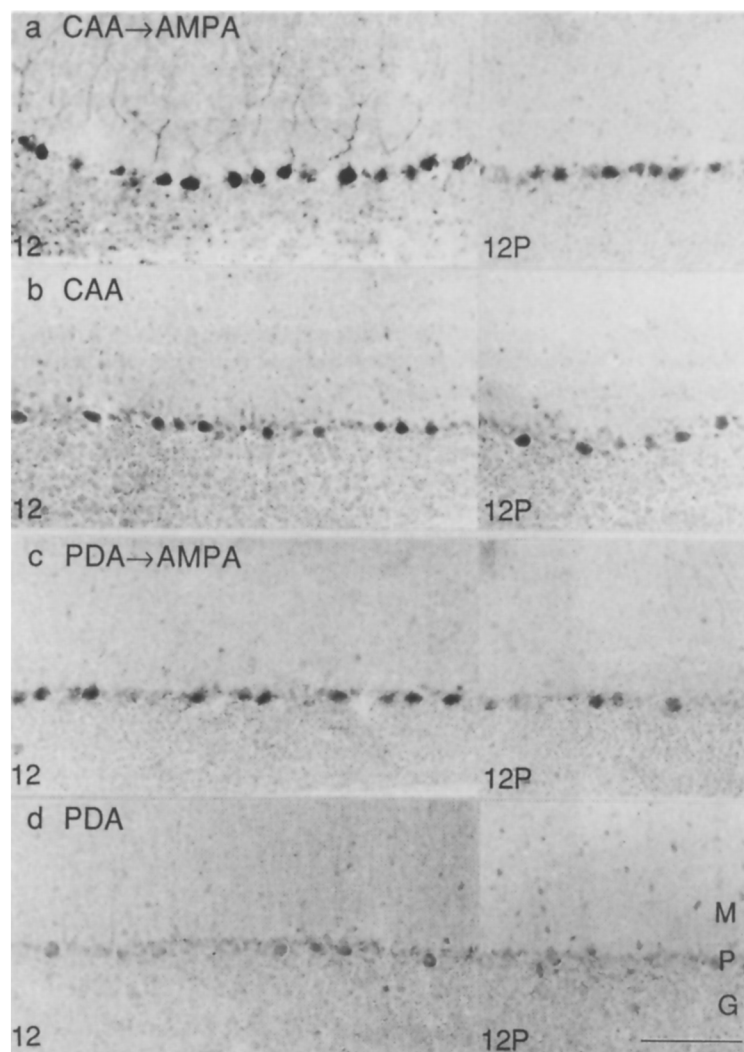
Figure 7. Effects of Calyculin A and PDA on 12P3-IR

(a and b) A 15 min calyculin A (CAA) pretreatment prior to a 4 min application of AMPA induced 12P3-IR in Purkinje cell dendrites (a), whereas CAA alone had only a small IR-inducing effect on the somata (b).

(c and d) A 15 min treatment with phorbol 12,13-diacetate (PDA) followed by a 4 min application of AMPA showed little effect on 12P3-IR on the Purkinje cell somata (c). PDA alone showed no effect on 12P3-IR (d) when compared with untreated slices (see Figure 3a). Adjacent, unlabeled panels have been reacted with 12P3 preadsorbed with peptide-12P (12P). Bar, 100 μm.

ished at 30 min compared with that in sections prepared from slices 5 min after treatment, the shafts of the distal dendrites were clearly stained, and prominent staining of the somata could still be observed. These findings were consistent with the immunoblot results showing a slight but detectable increase in 12P3 immunorecognition of the 105 kDa protein (see Figure 2B, lane 3). Actually, intracellularly recorded excitatory postsynaptic potentials evoked by parallel fiber stimulation clearly showed LTD-like sustained depression following treatment with 8-Br-cGMP (0.5 mM; 15 min) and then AMPA (10 μM; 4 min) in the presence of 1 μM BAPTA-AM without TTX; this is nearly the same condition as that causing long-term desensitization, suggesting the validity of this pharmacological protocol for induction of long-term desensitization. While 1 μM BAPTA-AM was added according to the previous protocol of Ito and Karachot (1992), no change was induced in parallel fiber-evoked potentials, suggesting that BAPTA-AM at this concentration does not interfere with parallel fiber-Purkinje cell transmission (Nakazawa et al., unpublished data). In contrast, 20 min after stimulation with AMPA alone, almost no 12P3-IR was detected in dendrites (Fig-

ure 6d). In sections from slices stimulated in the reverse order (AMPA for 4 min and then 8-Br-cGMP for 15 min) and fixed 30 min thereafter, 12P3-IR of Purkinje cell dendrites was never observed, whereas the Purkinje cell somata were still immunoreactive (Figure 6e). This protocol did not elicit sustained depression of excitatory postsynaptic potentials at parallel fiber-Purkinje cell synapses (Nakazawa et al., unpublished data).

The dependence of persistent phosphorylation on the type of cyclic nucleotide was examined using another membrane-permeable derivative of cGMP, dibutyryl-cGMP (dB-cGMP; 0.5 mM) as well as the cAMP equivalent of 8-Br-cGMP, 8-bromo-cAMP (8-Br-cAMP; 0.5 mM). Application of dB-cGMP for 15 min, followed by a 4 min AMPA stimulation, produced 12P3-IR in the dendrites for 30 min (Figure 6f). Application of 8-Br-cAMP followed by AMPA produced much less 12P3-IR at 30 min (Figure 6g). Application of 8-Br-cGMP, dB-cGMP, or 8-Br-cAMP alone produced no 12P3-IR in sections from slices fixed at 5 or 30 min after the application (result for 30 min after 8-Br-cGMP shown in Figure 6c). These results indicate that the cGMP pretreatment before AMPA application was required to
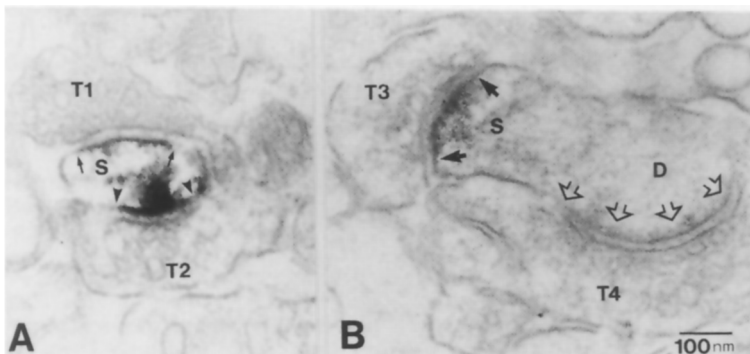
Figure 8. Localization of Immunoreactive AMPA Receptor Phosphorylation in the Purkinje Cell Dendritic Spine

Electron micrographs revealed immunoreactive profiles in the molecular layer of a cerebellar slice fixed 5 min after AMPA application for 4 min.

(A) Strong 12P3-IR was found in postsynaptic densities (PSDs) for T1 and T2 parallel fiber terminals between arrows and arrowheads, respectively.

(B) For a T3 parallel fiber terminal, the PSD (between arrows) showed 12P3-IR, whereas the PSD (open arrows) for a presumed climbing fiber terminal (T4) had no IR.

D, dendritic shaft; S, dendritic spine; T1–T4, presynaptic terminals.

produce a persistent phosphorylation of AMPA receptors in the dendrites.

As far as we can determine by light microscopy, application of a membrane-permeable phorbol ester, phorbol 12,13-diacetate (PDA; 0.2 μM) for 15 min, followed by a 4 min AMPA stimulation, did not produce 12P3-IR in Purkinje cell dendrites at 30 min after the application (Figure 7c). This protocol showed about the same duration of 12P3-IR as with AMPA alone (data not shown). PDA alone also showed no effect on 12P3-IR (Figure 7d). These findings suggest that the PKC activated by PDA (Castagna et al., 1982) is not involved in the persistent phosphorylation of Ser-696 in GluR2.

Effect of Protein Phosphatase Inhibitor on AMPA Receptor Phosphorylation

Increasing phosphorylation by blocking dephosphorylation in cerebellar long-term desensitization has been proposed by Ito and Karachot (1992) based on results using protein phosphatase (PPase) inhibitors. To examine whether PPase would affect AMPA receptor phosphorylation, a potent PPase 1 and 2A inhibitor, calyculin A (CAA; Bialojan and Takai, 1988), was applied to cerebellar slices prior to AMPA treatment. Application of 0.5 μM CAA for 15 min followed by AMPA stimulation for 4 min, one of the stimulating protocols causing long-term desensitization (Ito and Karachot, 1992), consistently induced persistent phosphorylation of AMPA receptor subunits in Purkinje cell dendrites (Figure 7a). CAA alone had a 12P3-IR-inducing effect on the somata but not on the dendrites (Figure 7b). Another line of evidence favoring the intracellular location of Ser-696 of GluR2 or corresponding sites of the other AMPA receptor subunits was the effect of an ecto-protein kinase inhibitor on the AMPA-induced phosphorylation. K252b, which is reported to be membrane impermeable (Nagashima et al., 1991), had very little effect on 12P3-IR when applied before and during AMPA stimulation for 4 min, even at the relatively high dose of 1 μM (data not shown). These results support the idea that AMPA receptor subunit phosphorylation is an equilibrium between protein kinases and phosphatases at the cytoplasmic side of the membrane (Ito and Karachot, 1992).

Localization of the Phosphorylated AMPA Receptors

Electron microscopic examination confirmed light microscopic findings that the immunoreactive product appeared in Purkinje cell dendrites of slices fixed 5 min after AMPA application. In electron micrographs, the electron opaque product was found to be exclusively associated with the postsynaptic densities (PSDs) of small and medium-sized synapses in the Purkinje cell dendrites (Figure 8). Small boutons forming single axospinous synapses (T1–T3) were considered to originate from parallel fibers. These electron-dense, immunoreactive products were concentrated inside the cell membrane, suggesting an intracellular localization of the phosphorylation sites. In contrast, a large bouton (Figure 8B, T4) probably originating from climbing fibers showed no 12P3-IR, whereas immunoreactivities of the C-terminal antibody of GluR1 and GluR2/3/4c have been reported in the postsynaptic specialization of both parallel and climbing fiber terminals (Baude et al., 1994; Nusser et al., 1994). Moreover, immunolabeling was rarely observed in equivalent areas of the molecular layer in untreated slices. No 12P3-IR was observed in ultrathin sections derived from an adjacent section of the AMPA-treated slices after preadsorption of 12P3 with phosphorylated peptide-12P, confirming the 12P3 immunospecificity at the electron microscopic level (data not shown). These results indicate that following stimulation with AMPA Ser-696 of GluR2, or the corresponding sites of other AMPA receptor subunits, is phosphorylated by intracellular kinases in Purkinje cell dendritic spines contacting parallel fibers.

Purkinje cell somata showed intense 12P3-IR more than 20 min after applied AMPA alone, whereas the dendritic IR had disappeared (see Figure 6d). This IR was not completely blocked by any of the kinase inhibitors used, except 1 μM staurosporine (see Figure 5) and 1 μM K252a (data not shown). Although some 12P3-IR was occasionally detected in the somata of interneurons, including stellate cells, basket cells, and Golgi cells, it was never observed in granule cells and Bergmann glial cells of any of the sections examined in these experiments. Since AMPA receptor subunits are expressed in both the granule cells

(GluR2, GluR4, and GluR4c, which is a subset of cerebellar GluR4; see Gallo et al., 1992) and Bergmann glial cells (GluR1, GluR4, and GluR4c; Keinänen et al., 1990; Gallo et al., 1992; Martin et al., 1993), this observation indicates that AMPA receptor phosphorylation occurs in a cell type–specific manner.

Discussion

In the present study, we examined the behavior of AMPA receptor phosphorylation and its spatial distribution in cerebellar slices at 5 and 30 min after different stimulating protocols, using a polyclonal antibody, 12P3, recognizing the phosphorylated Ser-696 in GluR2 or the homologous phosphorylated sites in GluR1/3/4.

The major findings in the present study are as follows. Subpopulations of cerebellar AMPA receptor subunits are phosphorylated in a cell type–specific manner by a brief AMPA exposure, whereas the subunits phosphorylated are not determined (see Figure 2; Figure 3). AMPA receptor subunits in Purkinje cell dendrites are rapidly phosphorylated by AMPA application alone (see Figure 4), but this phosphorylation is transient, disappearing within about 20 min (see Figure 6d). With pharmacological protocols producing long-term desensitization, 12P3-IR persisted for more than 30 min (see Figures 6a, 6b, and 6f; Figure 7a). Another protocol using PDA followed by AMPA, which also produces long-term desensitization, did not produce a persistent IR (see Figure 7c). The 12P3-IR was not inhibited by the available PKC or PKG inhibitors, but was inhibited by nonspecific kinase inhibitors (see Figure 5). Ser-696 of GluR2 or the homologous sites in GluR1/3/4 to be phosphorylated by AMPA application are located in the intracellular PSDs (see Figure 8).

Subpopulations of Cerebellar AMPA Receptor Subunits Are Phosphorylated

Results from immunoblots showing that some of the AMPA receptor subunits obtained by cerebellar homogenization are phosphorylated following AMPA bath application and results from immunohistochemistry demonstrating the predominant expression of 12P3-IR in Purkinje cells lead us to believe that cerebellar AMPA receptor phosphorylation occurs in a subunit type– and/or cell type–dependent manner. However, from our present study, it seems difficult to identify the subunit type phosphorylated, because 12P3 not only detects phosphorylation of Ser-696 in GluR2 but also may react with phosphorylated Ser-692 in GluR1, Ser-700 in GluR3, and Ser-687 in GluR4(c), owing to amino acid sequence similarities around the Ser residues. Another rat AMPA-type GluR subunit sequence, named GluRA (Keinänen et al., 1990), differs by 3 amino acids from GluR1, 1 of which is a Thr residue in place of Ser at 692. Since a synthetic peptide-12 from GluRA was phosphorylated by PKC (Nakazawa et al., submitted), the Thr-692 in GluRA is another possible recognition site for 12P3. Immunohistochemistry results demonstrating the predominant expression of 12P3-IR in Purkinje cells support the idea that the marked 12P3-IR bands in the immunoblots

are most likely due to the receptor subunits from Purkinje cells, which are GluR1–GluR3 (or A–C) but not GluR4 (or D) and GluR4c (Petralia and Wenthold, 1992; Gallo et al., 1992; Martin et al., 1993; Baude et al., 1994). Nevertheless, a smaller portion of the phosphorylated subunits could be located in cells other than Purkinje cells because 12P3-IR expression was occasionally seen in stellate, basket, and Golgi cells. Since granule cells express subunits 2, 4, and 4c, Bergmann glial cells express subunits 1, 4, and 4c, stellate/basket cells express subunits 3 and 4, and Golgi cells express subunit 3 (Petralia and Wenthold, 1992; Gallo et al., 1992; Martin et al., 1993; Baude et al., 1994), a possibility arises that 12P3 recognizes not only GluR2 but also GluR3. Actually, our finding showing phosphorylation of GluR2, GluR3, or GluR4c immunoprecipitated with the C-terminal antibody following glutamate treatment (see Figure 2C) does not contradict this possibility. Further study is necessary to distinguish which receptor subunits are phosphorylated in which cell types.

AMPA Receptors in Purkinje Cell Dendrites Are Transiently Phosphorylated by AMPA

While in naive slices a low basal level 12P3-IR was present only in the Purkinje cell somata, both somata and dendrites exposed to a brief AMPA application showed a prominent 12P3-IR within 2 min (see Figure 3; Figure 4), which was no longer detected after about 20 min (see Figure 6d). These results are in agreement with a recent report demonstrating synaptic activity–dependent GluR1 subunit phosphorylation using cortical cell cultures (Blackstone et al., 1994). The involvement of a kinase was shown by results obtained with staurosporine and K252a (see Figure 5), both of which are nonspecific inhibitors since they inhibit ATP binding to kinases (Herbert et al., 1990). Simultaneous application of the selective PKC and PKG inhibitors, calphostin C (0.5 μM) and KT5823 (0.5 μM), and even BAPTA-AM at 10 μM, failed to inhibit 12P3-IR in the dendrites. From these results, it seems reasonable to speculate that the phosphorylation of Ser-696 in GluR2 or of the homologous sites in GluR1/3/4 is due to the action of multiple kinases or a novel kinase that may be Ca^{2+} independent.

Agonist-induced phosphorylation of nicotinic acetylcholine receptor subunits has recently been suggested to involve a novel protein kinase (Miles et al., 1994). Ionic influx through activated AMPA receptor channels is also proposed to activate endogenous kinases (Lanius et al., 1993) and to be involved in cerebellar LTD induction in cultured Purkinje cells (Linden et al., 1993). The results presented here, along with the above cited results, suggest a signal transduction whereby the AMPA receptor binding its agonist becomes phosphorylated, perhaps to regulate its own functional activity (also see below).

Possible Function of Agonist-Induced AMPA Receptor Phosphorylation

In grease-gap recordings from wedges of cerebellum, the responses did not change amplitude with pulses of AMPA

applied repetitively at 10 min intervals (Ito and Karachot, 1992). According to these wedge results, the transient Ser-696 phosphorylation of GluR2 with applied AMPA would seem to have no cumulative effect on receptor function. It is to be noted, however, that a transient potentiation of AMPA receptor–dependent synaptic currents, correlated with the level of phosphorylation of their PSDs, is seen in hippocampal CA1 pyramidal cells (Wyllie and Nicoll, 1994). A number of reports also suggest that agonist-induced phosphorylation of both iono- and metabotropic receptor types is involved in their desensitization (for review, see Benovic et al., 1988; Huganir and Greengard, 1990), including the β_2-adrenergic receptor (Sibley et al., 1986), the nicotinic acetylcholine receptor (Hoffman et al., 1994), and the NMDA receptor (Tong et al., 1995).

In cerebellar slices from rats in which climbing fibers were selectively lesioned by 3-acetylpyridine, conjunctive stimulation of both parallel fibers and white matter elicited a transient (20–30 min) depression of the response (Shibuki and Okada, 1991). Since the majority of climbing fibers were absent in these slices, it would appear that the parallel fiber stimulation alone can induce short-term desensitization. However, in this case, glutamate released by the parallel fiber stimulation would activate AMPA receptors as well as the metabotropic glutamate receptors (mGluR1α), thus leading to PKC activation in the cytoplasm (Masu et al., 1991; Nusser et al., 1994). The transient phosphorylation obtained with AMPA alone may be one of the steps leading to changes in receptor channel function.

Persistent Phosphorylation of Purkinje Cell AMPA Receptors and Cerebellar Long-Term Desensitization

The three long-term desensitization protocols increasing intracellular cGMP (see Figures 6a and 6f) and that preventing dephosphorylation (see Figure 7a) prior to AMPA binding to the receptor resulted in persistent 12P3-IR in Purkinje cell dendrites. A 4 min, 100 μM quisqualate application causing long-term desensitization (Ito and Karachot, 1989) also showed persistent 12P3-IR in Purkinje cell dendrites (unpublished data). These results suggest that the sustained Ser-696 phosphorylation of GluR2 is correlated with long-term desensitization. Since LTD-like depression is also induced by pairing intracellular injections of cGMP or 8-Br-cGMP with parallel fiber stimulation (Daniel et al., 1993; Hartell, 1994), cGMP-primed, sustained phosphorylation at Ser-696 of GluR2 would also appear to be involved in cerebellar LTD. However, Ser-696 phosphorylation alone may not be sufficient for LTD induction because quisqualate-induced long-term desensitization (Ito and Karachot, 1990) and LTD-like depression (Hartell, 1994) are prevented by the selective PKG inhibitor, KT5823.

Another long-term desensitization–inducing protocol, PDA followed by AMPA, produced only a transient 12P3-IR (see Figure 7c), suggesting that 0.2 μM PDA has little effect on AMPA-induced Ser-696 phosphorylation of GluR2 at the light microscopic level. Since PKC activation is required for long-term desensitization (Ito and Karachot,

1992) and for LTD-like depression (Crepel and Krupa, 1988; Linden and Connor, 1991; Hartell, 1994), the lack of effect with 0.2 μM PDA may be due simply to an insufficient sensitivity of 12P3-IR to detect Ser-696 phosphorylation of GluR2 by PKC. Alternatively, cerebellar LTD may require phosphorylation of PKC-dependent sites other than the one recognized by 12P3. Direct phosphorylation of AMPA receptor subunits (GluR1–GluR4) by PKC has already been reported using another phorbol ester, 12-O-tetradecanoylphorbol 13-acetate, in cultured hippocampal neurons (Tan et al., 1994). Further study will be required to establish a causal relationship between the persistence of the receptor phosphorylation and cerebellar LTD.

The intense 12P3-IR observed in AMPA-stimulated slices was rather stable and long-lasting in Purkinje cell somata compared with the IR in the dendrites. This difference in duration may be due to different dephosphorylation kinetics. One of the PPases, PPase-1γ1, is localized over all the cytoplasmic faces of Purkinje cell spines and dendritic arbors as well as in the somata, whereas PPase-2A is homogeneously located in the somata and the thick dendrites, according to the results obtained using subtype-specific PPase antibodies (Hashikawa et al., 1995).

Membrane Topology of AMPA-Type GluR Subunits

Our electron micrographs using 12P3 suggest that Ser-696 of GluR2 is located intracellularly (see Figure 8). The images showed that the strong 12P3-IR was limited to the PSDs of Purkinje cell dendritic spines, and only weak IR was observed in the dendritic shaft (data not shown). Further supportive evidence for an intracellular localization of the site comes from the following experiments. Prolonged phosphorylation was obtained when CAA, a selective inhibitor of intracellular PPase-1 and PPase-2A, was preapplied prior to AMPA application, suggesting that dephosphorylation occurs on the cytoplasmic side (see Figure 6a). A nonpermeate ecto-protein kinase inhibitor, K252b, had no effect on 12P3-IR. The use of detergent (Triton X-100) facilitated the immunostaining of 12P3 on fixed slices (data not shown). Although it is suggested that immunocytochemical localization of peptide epitopes at the electron microscopic level is not conclusive to determine the topological map of membrane-integrated proteins (see Bennett and Dingledine, 1995), these results point to an intracellular location of phosphorylation at Ser-696 of GluR2 on the AMPA receptor. If the site did indeed face the cytoplasm, it should have shown a resting level IR. However, 12P3-IR was rarely observed at the electron microscopic level in the absence of agonist. A possible explanation for this threshold behavior of 12P3-IR with AMPA may be that Ser-696 of GluR2 is exposed for phosphorylation into the cytoplasmic side by a conformational change following AMPA binding. The alternative would be that GluR binding of AMPA triggers the activation of a kinase by ionic influx through activated AMPA receptor channels (Lanius et al., 1993; Linden et al., 1993).

Several recent reports have concluded that the entire region between TM3 and TM4 is extracellular (Wo and Oswald, 1994; Hollmann et al., 1994; Stern-Bach et al., 1994; Bennett and Dingledine, 1995; also see review by

Wo and Oswald, 1995). Although our present results do not resolve this question, our apparently intracellular receptor phosphorylation may be explained if the membrane topology of GluR subunits is different in a cell type– and/or subunit-specific manner. Alternatively, our results may provide evidence suggesting a dynamic change of GluR subunit membrane topology, i.e., an activity-dependent translocation of an amino acid segment between TM3 and TM4.

Experimental Procedures

Slice Preparation and Pharmacological Stimulation

Cerebellar vermi were dissected from male Wistar rats (210–260 g; 7–9 weeks old; SLC Inc., Japan) under ether anesthesia and parasagittally cut into 400 μm thick slices using a microslicer. Slices were immediately immersed on a nylon mesh in 200 ml of Krebs' solution saturated with 95% O_2/5% CO_2 and incubated at 33°C–34°C for 40 min. To avoid possible variations due to spontaneous activity, as in the wedge recording (Ito and Karachot, 1990), 0.5 μM TTX (Sankyo) and 1 μM BAPTA-AM (Dojin) were added to the medium until fixation. Each slice was then transferred to a cylinder chamber (3 cm diameter, 10 ml volume) in the same conditions as before, except that the medium contained one of the following: 10 μM AMPA (Research Biochemicals, Inc.), 0.5 mM 8-Br-cGMP (Sigma) or dB-cGMP (Sigma), 0.5 μM CAA (Wako Chemical Co.), or 0.2 μM PDA (LC Laboratories). Transferring the slices to different chambers for different times subjected the slice to different protocols as described previously (Nakazawa et al., 1993). The experimental protocols for long-term desensitization (Ito and Karachot, 1992) were 8-Br-cGMP followed by AMPA, dB-cGMP followed by AMPA, CAA followed by AMPA, and PDA followed by AMPA. The 8-Br-cGMP, dB-cGMP, CAA, or PDA was applied for 15 min and AMPA for 4 min. The protocols that do not elicit long-term desensitization were AMPA alone, AMPA followed by 8-Br-cGMP, and 8-Br-cAMP followed by AMPA. Protein kinase inhibitors such as K252a, K252b, KT5823, calphostin C (all from Kyowa Medex Co.), staurosporine (Sigma), CAA, PDA, and CNQX (Tocris Neuramin) were dissolved in dimethylsulfoxide and bath applied during slice incubation. The final concentration of dimethylsulfoxide in the incubation media was 0.1% (v/v), which did not affect 12P3-IR. At 5 or 30 min after incubation in Krebs' solution, the slices were treated with appropriate fixatives for light or electron microscopic observations. Postincubation times of 60 min gave variable results for the same protocols and are not reported here.

Immunoblotting

Cerebellar slices were homogenized in a 50 mM Tris–HCl (pH 7.4) buffer containing a cocktail of PPases and proteinase inhibitors (0.5 μM microcystin-LR [Calbiochem], 0.1 M β-glycerophosphoric acid, 15 mM sodium pyrophosphate, 50 mM sodium fluoride, 25 mM benzamidine, 1 mM EDTA, 0.5 mM EGTA, 2 μg/ml antipain, 2 μg/ml aprotinin, 2 μg/ml chymostatin, 2 μg/m pepstatin A) to avoid dephosphorylation and degradation of proteins, and solubilized by adding 4 × SDS–PAGE sample buffer containing 20 mM dithiothreitol. After gentle sonication, SDS–PAGE (7.5% acrylamide) was performed essentially as described elsewhere (Laemmli, 1970), and proteins were transferred to PVDF membrane (Immobilon-P, Millipore) by electroblotting (40 V overnight at 4°C; Towbin et al., 1979) using a transfer buffer (10 mM MOPS, 4 mM sodium acetate [pH 7.5], 20% ethanol, 0.1% SDS; Kidd et al., 1989). Blots were blocked for 2 hr with 3% bovine serum albumin in Tris-buffered saline at room temperature and then incubated for 2 hr at 37°C with 12P3, which was preadsorbed with 50 ng/ml peptide-12 or peptide-12P and then diluted to 50 μg/ml immunoglobulin (1:300 dilution) in 1% bovine serum albumin, 0.05% Tween-20 in Tris-buffered saline (TTBS). After washing with TTBS, blots were incubated with horseradish peroxidase– or alkaline phosphatase–conjugated goat anti-rabbit IgG (1:5000; Bio-Rad). Following several washes with TTBS, immunoreactive proteins were visualized with a Konica Immunostaining HRP-1000 (Konica, Tokyo, Japan) or alkaline phosphatase substrate, BCIP/NBT (Boehringer). For reprobing membranes, the blots were submerged in 0.1 M glycine–HCl (pH 2.0), blocked, and

restained by an antibody again. Anti-peptide-12 antibody, which was generated in rabbits immunized with a peptide corresponding to amino acids 690–701 of rat GluR2 (peptide-12; 690-VARVRKSKGKYA-701; diluted to 2 μg/ml) was also used as primary antibody for detecting GluR1–GluR4. The specificity of this antibody was confirmed by elimination of broad 97–106 kDa bands (see Figure 2) following preadsorption with peptide-12 (data not shown). A polyclonal antibody against the C-terminus of rat NMDAR1 (AB1516, Chemicon) was also used at a final concentration of 0.5 μg/ml.

Membrane Preparation and Immunoprecipitation

Crude membrane fraction from rat cerebellar slices was prepared as described previously (Lynch et al., 1982) with modifications. Briefly, three slices were homogenized in 300 μl of 0.32 M sucrose/distilled water with the cocktail of PPase and proteinase inhibitors described above, and with 0.1 μM staurosporine to inhibit kinases during homogenization. The homogenate was centrifuged at 534 × g for 4 min, and the supernatant was transferred to a 1.5 ml polycarbonate tube and centrifuged at 108,920 × g for 5 min (TL-100; Beckman). The membrane pellet was washed twice by resuspension in 100 μl of 5 mM Tris–HCl (pH 8.1) containing the cocktail of PPase and proteinase inhibitors and centrifuged at 108,920 × g for 5 min. The pellet was finally resuspended in 40 μl of 50-mM Tris–HCl (pH 7.4) and stored at −80°C until use.

Immunoprecipitation of GluR2/3/4c receptor subunits was carried out using the C-terminal antibody. This antibody directed against a synthetic peptide from the C-terminus of GluR2 (EGYNVYGIESVKI; the difference of the C-terminal 13 amino acid sequence in GluR3 or GluR4c from this sequence is only 1 amino acid; see Gallo et al., 1992) was prepared as described previously (Wenthold et al., 1992) and affinity purified on columns prepared by coupling the C-terminal peptide to activated-CH Sepharose 4B. The specificity of the antibody was almost the same as that of Ab25 (Wenthold et al., 1992) by immunoblot analysis, immunoprecipitation, and immunohistochemistry. The homogenates of membrane fractions from slices treated with 0.5 mM L-glutamate or from untreated slices were solubilized at 2.3 mg/ml protein in a solubilization buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 10 mM Tris–HCl [pH 7.4], 1 mM EDTA, with the cocktail of PPase and proteinase inhibitors described above) and incubated for 1 hr. The mixture was incubated for 1 hr with protein G–Sepharose 4FF (50% [v/v] in solubilization buffer), and bound impurities were then removed by brief centrifugation. C-terminal antibody (10 μg) diluted with PBS/1% Triton X-100 was added to 40 μg of the resulting membrane fraction supernatant, and samples were incubated for 3 hr at 4°C in a rotating apparatus at 15 rpm. Protein G–Sepharose 4FF was then added (0.3 ml of bed volume per milligram of IgG), and incubation was continued for 90 min. The protein G–Sepharose, separated by brief centrifugation, was washed three times with solubilization buffer containing 0.1% Triton X-100. Antibody–GluR complexes (the pellet fraction) were eluted from the protein G–Sepharose by boiling in SDS–PAGE sample buffer containing 20 mM dithiothreitol and examined by SDS–PAGE and immunoblot analysis using 12P3 preadsorbed with peptide-12. The protein content was assayed using the bicinchoninic acid method (Pierce). All procedures for membrane protein immunoprecipitation were carried out at 4°C.

Immunohistochemistry Protocols

The slices subjected to the protocols were fixed for 2 hr at 4°C, cryoprotected with 20% (w/v) sucrose in 0.1 M phosphate buffer (PB; pH 7.4) for several hours at 4°C, and further cut on a cryostat into parasagittal sections of 20 μm thickness. Sections were thaw mounted onto 3-aminopropyltriethoxysilane (Aldrich)–coated glass slides and stored at −80°C until use. Immediately prior to use, 12P3 was preadsorbed with peptide-12 or peptide-12P (50 ng/ml) for 6 hr at room temperature. The mounted sections were cool air dried, preincubated overnight at 4°C with 10% normal goat serum diluted with 0.1% Triton X-100/PBS, and again incubated overnight at 4°C with 12P3 at a concentration of 100 μg/ml IgG (1:150 dilution) in 0.1% Triton X-100/3% normal goat serum/PBS. Subsequently, sections were rinsed in PBS, incubated for 2 hr at room temperature with biotinylated goat anti-rabbit IgG (Vector) diluted at 1:200, rinsed in PBS, and placed for 1 hr in a solution containing avidin-biotin-peroxidase complex (Vector) diluted 1:100. After the final wash, color was developed for 12–18 min with 0.1 M PB

(pH 7.4) containing 0.005% 3,3'-diaminobenzidine tetrahydrochloride, 0.0025% nickel chloride, 0.0025% cobalt acetate, and 0.0012% hydrogen peroxide.

Electron Microscopic Immunohistochemistry

At 5 min after application of 10 μM AMPA, as described above, cerebellar slices were fixed with 4% paraformaldehyde/0.2% picric acid in 0.1 M PB (pH 7.4) for 1 hr and then left overnight in PB containing 20% sucrose. Parasagittal sections (10 μm) were cut on a cryostat (Frigocut 2800, Reichert-Jung) and mounted on glass slides. Immunocytochemistry was performed on these sections using the avidin-biotin-peroxidase complex method as described above, except for the use of 0.02% instead of 0.1% Triton X-100. Antibody 12P3 preadsorbed with peptide-12 or peptide-12P (50 ng/ml) was used as a primary antibody. After diaminobenzidine reaction, sections were osmicated (1% OsO₄ solution), dehydrated in graded ethanol, and covered with Epoxy resin (Fluka), which was allowed to polymerize at 60°C for 48 hr. Ultrathin sections from areas of the molecular layer were cut and stained with 1% uranyl acetate solution and observed with a JEOL 1200Ex electron microscope.

Acknowledgments

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