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Two Pathways of Cyclic GMP Production Through Glutamate Receptor-Mediated Nitric Oxide Synthesis

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Abstract: The selective agonists for the metabotropic glutamate receptor and the ionotropic non-*N*-methyl-D-aspartate (NMDA) glutamate receptor, (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (ACPD) and (*R,S*)-α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), respectively, increased the cyclic GMP (cGMP) content in cerebellar slices prepared from adult rats. The ACPD-induced rise in cGMP level was blocked by compounds known to antagonize metabotropic glutamate receptors, such as DL-2-amino-3-phosphonopropionic acid and L-2-amino-4-phosphonobutyric acid, but not by ionotropic glutamate receptor antagonists, D-2-amino-5-phosphonovaleric acid and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), whereas the AMPA-induced rise in cGMP level was suppressed by CNQX. Both rises in cGMP level involved nitric oxide synthase (NOS), because N^G-methyl-L-arginine (NMLA), an inhibitor of NOS, blocked both cGMP level rises, and excess L-arginine reversed the effect of NMLA. After lithium chloride treatment, which could exhaust phosphatidylinositol phosphates, ACPD no longer increased cGMP levels, whereas AMPA was still effective. In a calcium-free medium, ACPD still induced a rise in cGMP level, whereas AMPA did not. When the molecular layer was isolated to determine the cGMP content separately from that in the rest of the cerebellar cortex, it was found that ACPD raised the cGMP level mainly in the molecular layer, whereas AMPA raised it in both sections. These results suggest that ACPD enhances the cGMP level through activation of NOS independently of extracellular calcium, most likely by calcium release from intracellular stores triggered by metabotropic glutamate receptors linked to phosphoinositide breakdown, whereas AMPA activates the enzyme by calcium entry from the extracellular space triggered by ionotropic non-NMDA glutamate receptors.
Key Words: Cyclic GMP—Metabotropic glutamate receptors—Nitric oxide synthase—Cerebellum—(±)-1-Aminocyclopentane-*trans*-1,3-dicarboxylic acid—(*R,S*)-α-Amino-3-hydroxy-5-methylisoxazole-4-propionate. **Okada D.** Two pathways of cyclic GMP production through glutamate receptor-mediated nitric oxide synthesis. *J. Neurochem.* **59,** 1203–1210 (1992).

It has been shown that glutamate (Garthwaite and Balázs, 1978) and its agonists, such as *N*-methyl-D-aspartate (NMDA) (Garthwaite et al., 1988) and kainate (Garthwaite et al., 1989), increase the cyclic GMP (cGMP) content in the cerebellum through activation of neural nitric oxide synthase (NOS), a calmodulin-dependent enzyme (Bredt and Snyder, 1990). Because the effect of these glutamate agonists depends on extracellular calcium (Garthwaite et al., 1988), it is likely that the Ca^{2+} required for NOS activation is supplied by calcium influx from the extracellular space during membrane depolarization evoked by ionotropic glutamate receptor activation.

The metabotropic glutamate receptors linked to phosphoinositide (PI) metabolism exist in abundance in the cerebellum (Masu et al., 1991) and can liberate calcium from intracellular stores (Sugiyama et al., 1987). Calcium imaging techniques have revealed that calcium level increases occur in the restricted area that depends on their underlying mechanisms. For example, the calcium level increase induced by PI metabolism is restricted to an area near inositol trisphosphate-releasing sites (Parker and Ivorra, 1990). On the other hand, the calcium level increase due to NMDA receptor activation by presynaptic activity is restricted to the postsynaptic spines (Müller and Conner, 1991). These observations suggest that Ca^{2+} levels increased by different mechanisms have distinct

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Abbreviations used: ACPD, (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid; AMPA, (*R,S*)-α-amino-3-hydroxy-5-methylisoxazole-4-propionate; AP3, 2-amino-3-phosphonopropionic acid; AP4, 2-amino-4-phosphonobutyric acid; AP5, 2-amino-5-phosphonovaleric acid; cGMP, cyclic GMP; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; IBMX, 3-isobutyl-1-methylxanthine; NMDA, *N*-methyl-D-aspartate; NMLA, N^G-methyl-L-arginine; NOS, nitric oxide synthase; PI, phosphoinositide.

localizations and hence can play distinct roles (Alkon and Rasmussen, 1988). Thus, the activation of metabotropic glutamate receptors is another possible mechanism of NOS activation in the cerebellum.

In the present study, cGMP production mediated by metabotropic glutamate receptor activation was investigated in adult rat cerebellar slices. It was found that a selective agonist of metabotropic glutamate receptors, (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (ACPD) (Palmer et al., 1989), raised the cGMP content through a novel mechanism of NOS activation, which was predominant in the molecular layer and which was independent of extracellular calcium. In contrast, the AMPA-induced increase in cGMP content occurred in both the molecular layer and the other parts of the cerebellar cortex and depended on extracellular calcium.

MATERIALS AND METHODS

Male Wistar–ST rats (7–11 weeks old) were anesthetized with diethyl ether, bathed in ice-cooled water, and decapitated. Cerebella were dissected, and parasagittal slices of vermis (400 μm thick) were prepared at 0°C using a vibrating slicer (Dosaka, Kyoto, Japan) and transferred to Krebs buffer (100 ml) at 34°C for preincubation. The buffer contained 124 m*M* NaCl, 5.0 m*M* KCl, 1.24 m*M* NaH_2PO_4, 1.3 m*M* $MgSO_4$, 2.4 m*M* $CaCl_2$, 26 m*M* $NaHCO_3$, and 10 m*M* glucose and was equilibrated with 95% O_2/5% CO_2. Usually four to six slices were prepared from one rat. One slice served as the positive control, using 3 m*M* sodium nitroprusside to obtain full activation of guanylyl cyclase. Because the sensitivity of the slices to drugs is highly dependent on the conditions of preparation, if this control showed a cGMP level rise of <300 pmol/mg of protein (mean ± SE; 346 ± 15 pmol/mg of protein), all the slices were discarded.

For drug incubation, one slice was transferred to a tube containing 1 ml of Krebs buffer with the drugs at an appropriate time and kept at 34°C. O_2/CO_2 gas was introduced through thin tubes. 3-Isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, and the agonists were added after 85 and 90 min of preincubation, respectively. After 5 min of reaction with the agonist, slices were transferred to glass microhomogenizers and homogenized in 0.2 ml of cold 20% trichloroacetic acid (Wako Chemicals, Japan). The homogenate was centrifuged at 2,000 *g* for 5 min.

The supernatant was washed four times with water-saturated diethyl ether and neutralized with a small aliquot of 1 *M* NaOH to pH 7.4 before determination of cGMP content using a radioimmunoassay kit (Amersham, Japan), in duplicate. The cGMP content increased linearly with time of agonist exposure, reached a maximum by 3 min, and then did not change with reaction periods up to 10 min (data not shown). The pellet was dissolved in 1 *M* NaOH, and a small aliquot was subjected to protein assay in duplicate by the method of Lowry et al. (1951) using bovine serum albumin as the standard. The cGMP content is expressed as picomoles per milligram of protein, in mean ± SE values. Student's *t* test was performed with Yukms Statistical Library I software (Yukms Corp.).

6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), D-2-amino-5-phosphonovaleric acid (D-AP5), and ACPD (an equimolar mixture of 1*S*,3*R* and 1*R*,3*S* enantiomers) were purchased from Tocris Neuramin (U.K.). (*R*,*S*)-α-Amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), 2-amino-3-phosphonopropionic acid (AP3), and L-2-amino-4-phosphonobutyric acid (L-AP4) were purchased from RBI (U.K.). Lithium chloride and EGTA were purchased from Wako Chemicals and Wako-Dojindo (Japan), respectively. H7 and HA1004 were purchased from Seikagaku Kogyo (Japan). Other chemicals were purchased from Sigma (U.S.A.).

RESULTS

Basal cGMP levels

During the preincubation period, the cGMP level increased transiently (significant at 15–60 min), even though the incubation medium contained neither glutamate agonist nor IBMX (Fig. 1). This basal cGMP content then decreased gradually, reaching a stable level: 29.9 ± 2.7 pmol/mg of protein (n = 4) at 90 min, 24.5 ± 7.0 pmol/mg (n = 4) at 120 min. The basal cGMP level is higher than that reported by Southam and Garthwaite (1991*a*) (2.94 pmol/mg of protein), who boiled slices to terminate the reaction, whereas trichloroacetate was used for termination in the present study. The difference in termination methods might explain, at least in part, the different basal cGMP levels.

The transient increase in basal cGMP level was inhibited by addition of 0.1 m*M* N^G-methyl-L-arginine (NMLA), a specific NOS inhibitor, during preincubation (Fig. 1). Therefore, this increase was due to a spontaneous nitric oxide release, probably evoked by endogenous agonists that increased intracellular calcium levels. The measurements of cGMP levels described below were all conducted after the basal cGMP level was stabilized by incubation for 90 min.

ACPD-induced rise in cGMP level

As shown in Fig. 2A and B, addition of ACPD (at 1 m*M*, for 5 min) raised the cerebellar cGMP content to 59.4 ± 2.8 pmol/mg (n = 22), corresponding to 179% of the basal level ($p < 0.001$). The effect of ACPD was

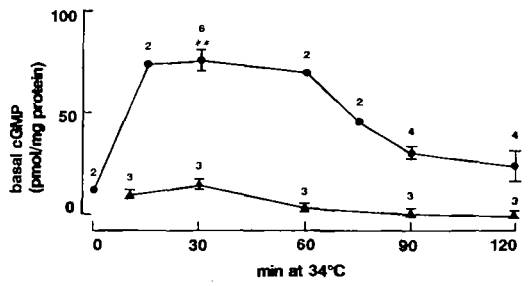

FIG. 1. Changes in the basal cGMP level during incubation without IBMX at 34°C: control buffer (●) and with 0.1 m*M* NMLA added at zero-time (▲). At zero-time, the slice immediately after the preparation at 0°C was transferred to the incubation buffer at 34°C, and the slices were incubated further for the periods indicated in the abscissa. Data are mean ± SE (bars) values from the indicated number of trials. $^{**}p < 0.01$ for difference from the 90-min incubation in control buffer.

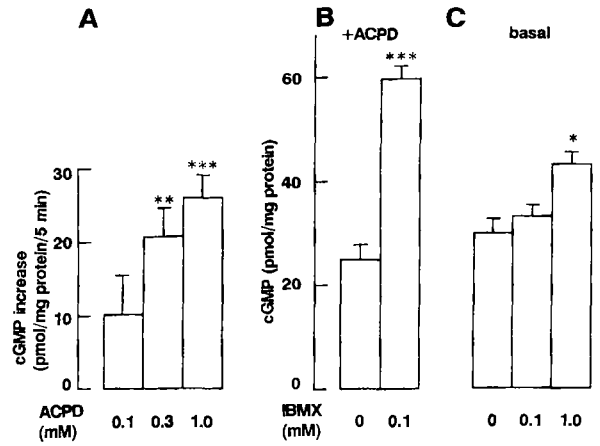

FIG. 2. A: The ordinate expresses the cGMP level increase above the basal level, in picomoles per milligram protein per 5 min, in response to ACPD. $^{**}p < 0.01$, $^{***}p < 0.001$ for difference from basal level. **B:** Effect of IBMX on the ACPD (1 m*M*)-induced rise in cGMP level. $^{***}p < 0.001$ for difference from the corresponding basal value shown in C (0.1 m*M* IBMX). **C:** Effect of IBMX on basal cGMP levels. $^{*}p < 0.05$ for difference from IBMX at 0 m*M*. Data are mean ± SE (bars) values.

dose dependent, as shown in Fig. 2A. ACPD at 0.3 m*M* was as effective as at 1 m*M* (n = 6, $p < 0.01$), but its effect was not significant at 0.1 m*M* (n = 8) (see also Southam and Garthwaite, 1991*b*). This dose–response relationship was similar to that of the ACPD-induced Cl^- current obtained from *Xenopus* oocytes expressing metabotropic glutamate receptors by injection of rat brain RNAs (Tanabe et al., 1991).

Ionotropic glutamate receptor antagonists did not affect the cGMP level rise induced by 0.3 m*M* ACPD (0.2 m*M* D-AP5, 57.5 ± 4.3 pmol/mg, n = 5; 0.1 m*M* CNQX, 51.3 ± 1.9 pmol/mg, n = 4), nor did they affect the basal cGMP levels (D-AP5, 30.6 ± 5.5 pmol/mg, n = 4; CNQX, 34.3 ± 0.5 pmol/mg, n = 4). These results (Fig. 3) show that the ionotropic glutamate receptors were not involved in the ACPD-induced rise in cGMP level.

Antagonistic effects of L-AP4 (Nicoletti et al., 1986) and DL-AP3 (Schoepp and Johnson, 1989) on the PI breakdown induced by metabotropic glutamate receptors have been reported. In contrast to D-AP5 and CNQX, 1 m*M* L-AP4 and AP3 significantly ($p < 0.001$) reduced the cGMP level rise induced by 0.3 m*M* ACPD to 32.5 ± 6.1 and 43.5 ± 3.7 pmol/mg (n = 6), respectively, as shown in Fig. 3. They did not alter the basal cGMP levels: L-AP4, 29.1 ± 7.3 pmol/mg, n = 6; AP3, 35.5 ± 2.7 pmol/mg, n = 4. These pharmacological properties resemble those of metabotropic glutamate receptors and strongly suggest that ACPD raises cGMP levels in adult rat cerebellar slices through metabotropic glutamate receptors.

Effect of IBMX

Figure 2C shows the effects of IBMX on the basal cGMP levels. When the slices were incubated with IBMX at 0.1 m*M* for 10 min, the basal cGMP level was not altered (33.2 ± 2.1 pmol/mg, n = 17), whereas at 1 m*M*, IBMX slightly increased the basal level (43.3 ± 2.2 pmol/mg, n = 6; $p < 0.05$). This dose–response relationship is similar to that of the IBMX-induced rise in cyclic AMP level in endothelial cells (Hong, 1983). Thus, IBMX at 0.1 m*M* has virtually no effect on the cGMP levels.

The cGMP level rise induced by 1 m*M* ACPD was not observed in the absence of IBMX (25.0 ± 2.9 pmol/mg, n = 4), as shown in Fig. 2B. This indicates that cerebellar tissue contains phosphodiesterase activities that prevent the sustained increase of cGMP levels. In all experiments except for those shown in Figs. 1, 2B, and 2C, the reaction mixture contained IBMX at 0.1 m*M*.

AMPA-induced rise in cGMP level

As shown in Fig. 4, AMPA at concentrations of >0.3 μM also induced a cGMP level rise in the adult rat cerebellum in a dose-dependent manner. In contrast to ACPD, the cGMP level rise induced by 0.3 or 1 μM AMPA was effectively suppressed by 0.1 m*M* CNQX: 0.3 μM AMPA plus CNQX, 28.3 ± 2.5 pmol/mg, n = 5. Therefore, the effect of AMPA was due to activation of non-NMDA ionotropic glutamate receptors. The AMPA-induced rise in cGMP levels also required 0.1 m*M* IBMX (data not shown). When AMPA and ACPD were applied simultaneously, there was no indication of any associative effects (data not shown).

Involvement of nitric oxide

Because agonist-induced rises in cGMP levels required the coexistence of low concentrations of IBMX, it is necessary to determine which process was responsible for the cGMP level rise: inhibition of phosphodiesterase or activation of guanylyl cyclase.

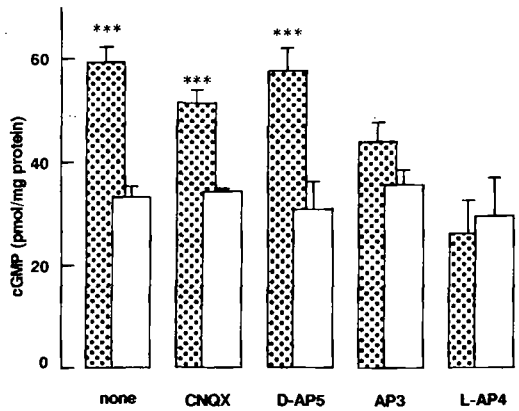

FIG. 3. Effect of glutamate receptor antagonists on the rise in cGMP levels induced by 0.3 m*M* ACPD: no antagonist, 0.1 m*M* CNQX, 0.2 m*M* D-AP5, 1 m*M* AP3, and 1 m*M* L-AP4. Dotted columns represent ACPD-induced cGMP levels. Data are mean ± SE (bars) values. $^{***}p < 0.001$ for difference from corresponding basal levels (without ACPD but with antagonist; open columns).

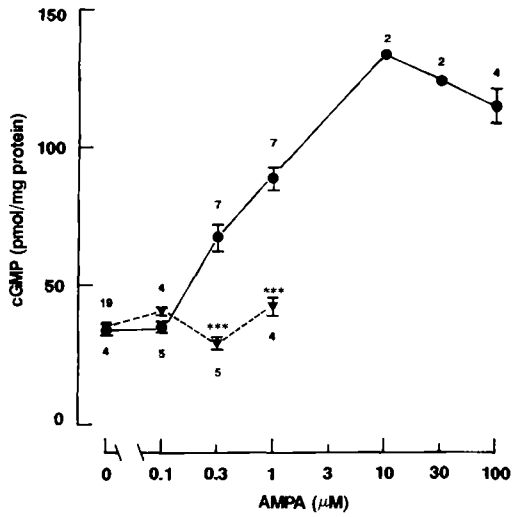

FIG. 4. Dose–response relationship of AMPA-induced cGMP production (●) and effect of 0.1 m*M* CNQX on the AMPA-induced rise in cGMP levels (▼). Data are mean ± SE (bars) values from the indicated number of trials. ***$p < 0.001$ for difference from the corresponding controls (without CNQX).

When the slices were incubated with 0.1 m*M* NMLA (plus 0.1 m*M* IBMX), a competitive inhibitor of NOS, for 10 min before addition of ACPD, the effect of ACPD was completely blocked (Fig. 5; 17.2 ± 6.0 pmol/mg, n = 5; $p < 0.01$), and the basal level was not changed (24.9 ± 5.5 pmol/mg, n = 5). The effect of NMLA was antagonized by 1 m*M* L-arginine, which is the physiological substrate for NOS, as shown in Fig. 5 (76.3 ± 8.8 pmol/mg, n = 4; difference from NMLA plus ACPD was significant at $p < 0.001$). NMLA plus L-arginine did not alter the basal level (39.4 ± 4.1 pmol/mg, n = 4). These results indicated that ACPD raised the cGMP content through activation of NOS.

The cGMP content rise induced by 0.3 μ*M* AMPA was also dependent on NOS. It was inhibited by NMLA (13.1 ± 2.2 pmol/mg, n = 5), and L-arginine antagonized the effect of NMLA (56.6 ± 3.5 pmol/mg, n = 5). Thus, both metabotropic and ionotropic glutamate receptor agonists activate NOS to increase the cGMP content in rat cerebellar slices. NOS produces nitric oxide, which activates guanylyl cyclase. Therefore, it is likely that the agonist-induced rises in cGMP levels are evoked through activation of guanylyl cyclase.

Involvement of PI metabolism

Metabotropic glutamate receptors linked to PI metabolism liberate calcium from intracellular stores (Sugiyama et al., 1987). The Ca^{2+} required for ACPD-induced NOS activation can thus be supplied by this mechanism. It is well known that lithium chloride inhibits inositol formation, and when PI turnover is active, this inhibition subsequently inhibits the reproduction of phosphatidylinositols (Berridge et al., 1982; Worley et al., 1988). The basal cGMP level is enhanced by spontaneous NOS activity during the preincubation period, as shown in Fig. 1, and this NOS activity should be evoked by endogenous agonists, such as glutamate. It is also well known that the cerebellum is enriched with PI metabolism triggered by glutamate (Hwang et al., 1990). Hence, it is likely that this transient increase in the basal cGMP level is accompanied by spontaneous PI turnover. Thus, it is expected that the lithium chloride treatment during the preincubation period exhausts the resource of inositol 1,4,5-trisphosphate and subsequently suppresses the action of ACPD on PI turnover.

Lithium chloride at 3 m*M* was added to the preincubation medium 1 h before addition of 1 m*M* ACPD. As shown in Fig. 6, lithium chloride significantly suppressed the effect of ACPD (31.7 ± 2.8 pmol/mg, n = 7; $p < 0.001$). The lithium treatment did not alter the basal level (25.6 ± 3.4 pmol/mg, n = 5). In contrast, the AMPA-induced rise in cGMP level was not affected by the same treatment (65.6 ± 2.2 pmol/mg, n = 4; Fig. 6B). These results support the hypothesis that an ACPD-induced rise in cGMP level involves PI breakdown, whereas the basal cGMP level and AMPA-induced rise in cGMP level are independent of PI breakdown.

Effect of calcium depletion

Metabotropic glutamate receptors may increase intracellular calcium levels through a pathway other than PI-induced liberation of calcium from intracellular stores because this receptor activates protein kinase C, which closes potassium channels (Baraban et al., 1985); the subsequently induced depolarization

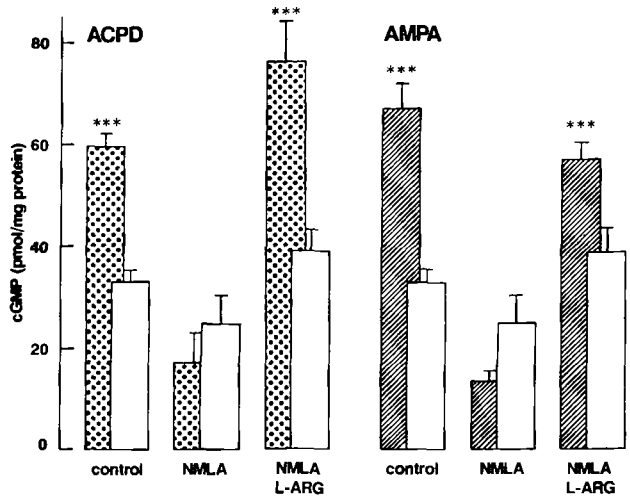

FIG. 5. Effects of NMLA (0.1 m*M*) and L-arginine (L-ARG; 1 m*M*) on the rise in cGMP level induced by 1 m*M* ACPD (▨) and 0.3 μ*M* AMPA (▨). Basal cGMP levels are also shown (□). The "control" data are identical with those in Figs. 2B and 4. Data are mean ± SE (bars) values. ***$p < 0.001$ for difference between basal and agonist-induced cGMP levels.

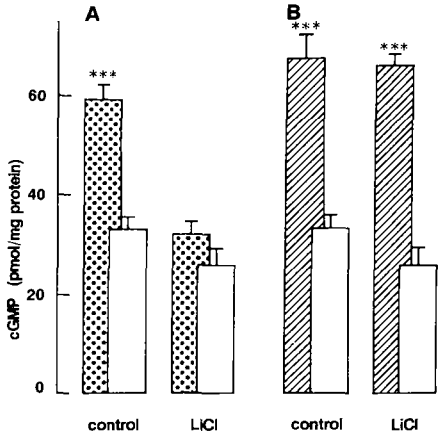

FIG. 6. Effect of incubation with 3 m*M* LiCl for 1 h on (**A**) 1 m*M* ACPD- and (**B**) 0.3 μM AMPA-induced rises in cGMP levels. Basal cGMP levels are also shown (□). The data without LiCl are from Figs. 2B and 4. Data are mean ± SE (bars) values. ***$p < 0.001$ for difference between agonist-induced and basal cGMP levels.

would enhance calcium influx through voltage-gated calcium channels. To test this possibility, an experiment was conducted using an extracellular medium depleted of Ca^{2+}.

The application of 1 m*M* ACPD to slices preincubated in a calcium-free medium (0.2 m*M* EGTA without $CaCl_2$ for 15 min) raised the cGMP content to levels similar to those in the normal calcium medium (63.5 ± 6.4 pmol/mg, n = 5), as shown in Fig. 7. The difference from the normal condition was insignificant. This result showed that the ACPD-induced rise in cGMP levels was independent of extracellular calcium and suggested the involvement of calcium release from intracellular stores.

In contrast, under the same calcium-depleted condition, the effect of AMPA at 0.3 μM was completely suppressed (Fig. 7; 24.5 ± 2.8 pmol/mg, n = 4), and the difference from the normal condition was significant ($p < 0.001$). This result indicates that, unlike the ACPD-induced rise in cGMP levels, the AMPA-induced rise depends on calcium influx from the extracellular space.

Layer analysis

In some experiments, the slices were put on glass slides immediately after reaction and fixed by microwave irradiation for 15 s (RE-M15 microwave oven, 500 W, 2.45 GHz; Sharp Co. Ltd., Japan). The molecular layer (including the Purkinje cell layer) was then isolated, and the cGMP content was measured separately for the molecular layer and the remainder (the granule cell layer plus white matter). The results are shown in Fig. 8.

In control slices (without agonists), the two parts showed similar basal cGMP contents: molecular layer, 33.5 ± 8.3 pmol/mg; remainder, 42.5 ± 11.5 pmol/mg (n = 3). The cGMP content of the entire slice was calculated from the amount of cGMP and protein in each region and was compared with the entire slice measurement. These values (entire slice observed, 33.2 ± 2.1 pmol/mg, n = 17; calculated, 38.3 ± 2.4 pmol/mg, n = 3) were similar, and the difference was not significant, suggesting that the enzymatic activities were terminated by the microwave irradiation and that there was no loss of cGMP after separation of the layers.

ACPD raised the cGMP content mainly in the molecular layer. The increase in cGMP level above the basal value in the molecular layer (obtained from the average of the ACPD-induced level minus that of the basal level in each part) was 55.5 ± 11.7 pmol/mg (n = 3), significantly larger than the 7.5 ± 4.4 pmol/mg (n = 3) of the remainder ($p < 0.05$). The calculated content of the entire slices matched the observed values: observed, 59.4 ± 2.8 pmol/mg, n = 22; calculated, 59.0 ± 2.6 pmol/mg, n = 3.

AMPA raised the cGMP content of both the molecular layer and the remaining part of the cerebellar slices. The increases above the basal level in the molecular layer and in the remainder were 64.0 ± 13.1 and 35.0 ± 18.4 pmol/mg (n = 5), respectively. Both agonists raised the cGMP content similarly in the molecular layer, whereas AMPA induced a larger rise than ACPD in the remainder. The calculated and observed contents of the entire slice were 77.2 ± 6.6 (n = 5) and 88.5 ± 4.4 (n = 7) pmol/mg, respectively.

DISCUSSION

Because no specific antagonist for the metabotropic glutamate receptor is available, various receptor antagonists were tested against the most specific agonist, ACPD (Palmer et al., 1989), to evaluate the involvement of this receptor. In addition, the involvement of

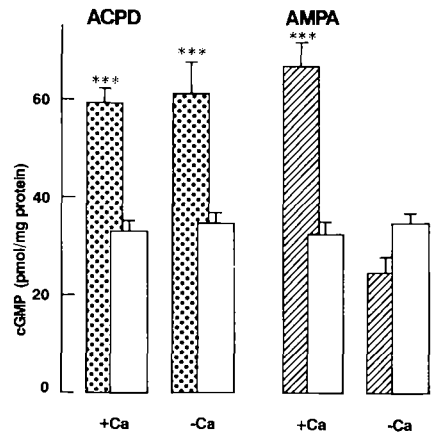

FIG. 7. Effect of calcium depletion on 1 m*M* ACPD- and 0.3 μM AMPA-induced rises in cGMP levels. Normal medium (denoted as +Ca) contained 2.4 m*M* $CaCl_2$, and calcium-depleted medium (denoted as −Ca) contained 0.2 m*M* EGTA without added $CaCl_2$. Basal cGMP levels are also shown (□). Data are mean ± SE (bars) values. ***$p < 0.001$ for difference between agonist-induced and basal cGMP levels.

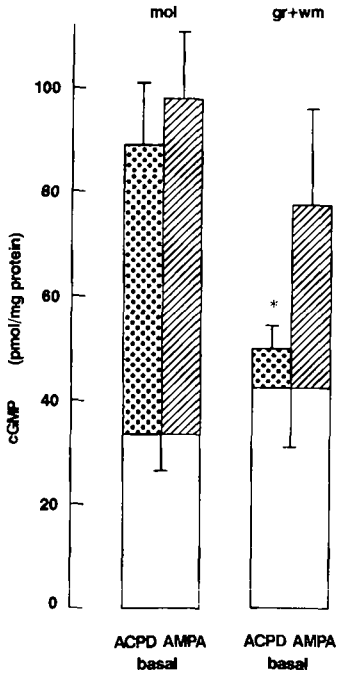

FIG. 8. Layer analysis in the molecular layer (mol) and the remaining part [granular cell layer plus the white matter (gr + wm)]. Basal cGMP levels are indicated (□) below the ACPD (▨)- and AMPA (▨)-induced increase in cGMP levels above the basal level. The ordinate shows the total cGMP levels. Data are mean ± SE (bars) values. One slice contained 1.3–2.0 (mean, 1.65) mg of protein. The molecular layer from one slice contained 0.32 mg of protein and 1.07 mg for the remainder (average protein yield, 84%). *$p < 0.05$ for difference between ACPD-induced increases in cGMP levels in the molecular layer and the remainder.

PI breakdown and the independence of extracellular calcium were studied. From this combination of experiments, the present results show that NOS is activated by the metabotropic action of glutamate receptors, triggered by ACPD, in a manner independent of extracellular calcium, most likely due to intracellular calcium liberation. Nitric oxide thus produced activates guanylyl cyclase and thereby enhances the cGMP content in the molecular layer. AMPA also raised the cGMP content in the cerebellum through activation of NOS, but this effect was dependent on extracellular calcium, suggesting the involvement of calcium entry.

The basal cGMP level after 90 min of preincubation is maintained, at least in part, by an equilibrium between NOS and phosphodiesterase activities, as judged from the effects of IBMX and NMLA (Figs. 2 and 5). The basal cGMP level might be maintained also by NMDA receptor activation, as Southam and Garthwaite (1991*a*) reported. In the present study, however, D-AP5 did not significantly alter the basal level (Fig. 3). Both AMPA- and ACPD-induced rises in cGMP levels required 0.1 m*M* IBMX. This requirement is well explained by the effect of IBMX as a phosphodiesterase inhibitor. First, 0.1 m*M* IBMX does not significantly affect the cGMP level itself (Fig. 2C). The agonist-induced rises in cGMP level are due to NOS activation but not to phosphodiesterase inhibition because the agonist-induced cGMP rise is inhibited by NMLA, an NOS inhibitor (Fig. 5). Second, Hong (1983) reported the effect of IBMX on phospholipases, which was obvious at concentrations of >1 m*M*, suggesting that 0.1 m*M* IBMX does not affect phospholipases. Third, Breer et al. (1990) showed rapid formation of cyclic AMP and inositol trisphosphates in olfactory transduction. These second messengers were degraded within some 100 ms. It is likely that the phosphodiesterase in the cerebellum also degrades cGMP produced in response to ACPD or AMPA very rapidly.

The involvement of PI breakdown in ACPD responses was indicated by blockade with lithium chloride and also through the dependence on extracellular calcium. The results of these experiments clearly discriminated the effects of ACPD and AMPA. The ACPD-induced rise in cGMP levels was suppressed by lithium chloride, but the AMPA-induced rise was not. From layer analysis, the ACPD-induced rise in cGMP level was observed mainly in the molecular layer. This distribution is consistent with autoradiographic demonstration of ACPD-induced PI turnover (Hwang et al., 1990). The ACPD-induced rise in cGMP level was not altered by incubation with 0.2 m*M* EDTA medium, whereas the AMPA-induced rise was completely reduced, showing that under the present conditions, calcium depletion did not affect the intracellular calcium stores but effectively reduced extracellular calcium activity. More vigorous conditions to deplete extracellular calcium will exhaust intracellular stores, and, in fact, incubation with a medium containing 0.3 m*M* EGTA for 20 min decreased the ACPD-induced rise in cGMP levels (data not shown). These observations suggest that in the ACPD-induced rise in cGMP levels, NOS is activated by intracellular calcium release.

The involvement of protein kinase C, whose activation leads to calcium entry (Baraban et al., 1985), is excluded from the ACPD-induced rise in cGMP levels, which occurs under calcium-free conditions. However, protein kinases can be involved in an ACPD-induced rise in cGMP levels as long as their action or activation is independent of calcium entry. In fact, protein kinase inhibitors (staurosporine at 0.5 μM for 15 min, H7 at 0.3 m*M* for 60 min, HA1004 at 0.3 m*M* for 60 min, and polymyxin B at 0.1 m*M* for 60 min), as well as a protein kinase C activator (phorbol 12,13-diacetate at 0.5 μM for 10 min), enhanced the basal cerebellar cGMP levels (author's unpublished data). These results suggest the interesting possibility that protein kinases can regulate the cerebellar cGMP level through modification of NOS, because the cloned NOS has putative phosphorylation sites (Bredt et al., 1991).

Thus, NOS is activated through two different pathways in cerebellar slices. One pathway is mediated by ionotropic glutamate receptors and depends on extra-

cellular calcium, whereas the other is initiated by metabotropic glutamate receptors and is dependent on PI breakdown but not on extracellular calcium. The former pathway of NOS activation reported previously (Garthwaite et al., 1988) is likely to involve calcium entry, whereas the latter pathway proposed in the present study most likely involves calcium liberation from intracellular stores. Inositol trisphosphate receptors are abundant in Purkinje cells, which explains most of the glutamate-activated increase in intracellular calcium levels in Purkinje cell dendrites (Llano et al., 1991), where voltage-dependent calcium channels also exist (Tank et al., 1988). In *Xenopus* oocytes, the increase in intracellular calcium levels induced by inositol 1,4,5-trisphosphate was restricted to within a few micrometers of the inositol 1,4,5-trisphosphate release site (Parker and Ivorra, 1990). On the other hand, possible clustering of calcium channels in the plasma membrane was also suggested (Silver et al., 1990). These differential localizations of the two machineries that regulate intracellular calcium suggest possible segregation of the two mechanisms, liberation and entry. The novel pathway for NOS activation will play an important role in NOS regulation.

Long-term depression of cerebellar synaptic transmission between parallel fibers and Purkinje cells is evoked by the simultaneous activation of parallel and climbing fibers. One of the roles of climbing-fiber activity in this synaptic plasticity is the release of nitric oxide followed by the elevation of cGMP levels (Shibuki and Okada, 1991). Long-term desensitization of the AMPA receptor is evoked by conjunctive application of AMPA with 8-bromo-cGMP or ACPD (Ito and Karachot, 1990) and is proposed to be the molecular event underlying long-term depression (Ito, 1990). These reports suggested a possible link between the metabotropic glutamate receptors and cGMP production. The present results are apparently consistent with this suggestion. However, the present study also revealed that AMPA alone produced cGMP in the cerebellar slices, although AMPA alone cannot evoke long-term desensitization (Ito and Karachot, 1990). One possible explanation of this discrepancy is the differential localization of AMPA- and ACPD-induced rises in cGMP levels suggested by the following observations: (a) Dumuis et al. (1990) reported associative effects of ionotropic and metabotropic glutamate receptors in the arachidonate release of the cultured striatal cells. However, in the present study no indication of any association was observed. (b) Vranesic et al. (1991) reported that the ACPD-induced increase in calcium levels in Purkinje cells was restricted to the soma, whereas AMPA receptors are expected to exist in fine dendrites of the cell because the postsynaptic receptor of parallel-fiber Purkinje-cell transmission is blocked by CNQX (Konnerth et al., 1990).

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