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The Inhibition of Glutamate Release by Metabotropic Glutamate Receptor 7 Affects Both $[Ca^{2+}]_c$ and cAMP

EVIDENCE FOR A STRONG REDUCTION OF Ca^{2+} ENTRY IN SINGLE NERVE TERMINALS*

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Metabotropic glutamate receptors (mGluRs) from group III reduce glutamate release. Because these receptors reduce cAMP levels, we explored whether this signaling pathway contributes to release inhibition caused by mGluRs with low affinity for L-2-amino-4-phosphonobutyrate (L-AP4). In biochemical experiments with the population of cerebrocortical nerve terminals we find that L-AP4 (1 mM) inhibited the Ca^{2+}-dependent-evoked release of glutamate by 25%. This inhibitory effect was largely prevented by the pertussis toxin but was insensitive to inhibitors of protein kinase C bisindolylmaleimide and protein kinase A H-89. Furthermore, this inhibition was associated with reduction in *N*-type Ca^{2+} channel activity in the absence of any detectable change in cAMP levels. In the presence of forskolin, however, L-AP4 decreased the levels of cAMP. The activation of this additional signaling pathway was very efficient in counteracting the facilitation of glutamate release induced either by forskolin or the β-adrenergic receptor agonist isoproterenol. Imaging experiments to measure Ca^{2+} dynamics in single nerve terminals showed that L-AP4 strongly reduced the Ca^{2+} response in 28% of the nerve terminals. Moreover, immunochemical experiments showed that 25–35% of the nerve terminals that were immunopositive to synaptophysin were also immunoreactive to the low affinity L-AP4-sensitive mGluR7. Then, mGluR7 mediates the inhibition of glutamate release caused by 1 mM L-AP4, primarily by a strong inhibition of Ca^{2+} channels, although high cAMP uncovers the receptor ability to decrease cAMP.

Metabotropic glutamate receptors (mGluR)[1] from group III consist of four different subtypes (mGluR4, -6, -7, and -8) and are activated by the selective agonist L(+)-2-amino-4-phosphonobutyrate (L-AP4) (Refs. 1–5; for review, see Refs. 6 and 7). The localization of these receptors within presynaptic active zones (8, 9) is consistent with their role as autoreceptors mediating the feedback inhibition of glutamate release (10–13). In neuronal preparations the inhibition of glutamate release by these receptors has been considered to be mediated by the reduction of voltage-dependent Ca^{2+} channel activity (11, 14, 15). However, these receptors also decrease cAMP levels both in heterologous expression systems (1–5) and in neuronal preparations (13, 16). Nevertheless, it remains unclear what influence this signaling pathway has on the inhibition of glutamate release.

The activity of L-AP4-sensitive mGluRs is developmentally regulated in different brain areas (13, 17–19). In the cerebral cortex mGluRs with high affinity for L-AP4 (EC_{50} 2.3 μM) potently reduce glutamate release in nerve terminals from young (13) but not from adult rats (18). It is therefore possible that in the cerebral cortex of adult rats, mGluRs with low affinity for L-AP4 act as presynaptic receptors that mediate synaptic inhibition. In preparations of this tissue, we have examined whether a mGluR with low affinity for L-AP4 reduces glutamate release in cerebrocortical nerve terminals from adult rats and, moreover, what role the decrease in cAMP levels plays in inhibiting release. Secondly, by combining Ca^{2+} imaging and immunocytochemistry we determined the impact of receptor activation on the Ca^{2+} dynamics of single nerve terminals to identify the type of mGluR that mediates this response. We found that a mGluR with low affinity for L-AP4 inhibits glutamate release by signaling through two pathways. In one pathway, inhibition of glutamate release occurs by reducing the activity of *N*-type Ca^{2+} channels in the absence of any change in cAMP. However, when cAMP levels are higher, the receptor is able to signal through another pathway that diminishes the levels of cAMP, thereby counteracting the facilitation of glutamate release by PKA activation. In addition, the L-AP4-sensitive low affinity mGluR was identified as mGluR7, which was found in a subpopulation of nerve terminals (25–35%), where its activation dramatically reduces depolarization-induced entry of Ca^{2+} and the ensuing release of glutamate.

EXPERIMENTAL PROCEDURES

Synaptosomal Preparation

Synaptosomes were purified on discontinuous Percoll (Amersham Biosciences) gradients as previously described (20, 21). Briefly, the

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[1] The abbreviations used are: mGluR, metabotropic glutamate receptor; L-AP4, L(+)-2-amino-4-phosphonobutyrate; PKC, protein kinase C; PKA, protein kinase A; CPPG, (*RS*)-α-cyclopropyl-4-phosphonophenylglycine; MPPG, (*RS*)-α-methyl-4-phosphonophenylglycine; PDBu, 4β-phorbol 12,13-dibutyrate; α-PDD, 4α-phorbol 12,13-didecanoate; CgTx-GVIA, ω-conotoxin-GVIA; Aga-IVA, ω-agatoxin-IVA; BSA, bovine serum albumin; Sp-8-Br-cAMPS, 8-bromoadenosine-3′,5′-cyclic monophosphothiorate S_p isomer; 4AP, 4-amino-pyridine;HBM, HEPES buffer medium; MOPS, 4-morpholinepropanesulfonic acid; TBS, Tris-buffered saline.

cerebral cortex was isolated from adult male Wistar rats (2–3 months) and homogenized in a medium containing 0.32 M sucrose, pH 7.4. The homogenate was centrifuged for 2 min at 2,000 × *g* at 4 °C, and the supernatant was spun again at 9,500 × *g* for 12 min. From the pellets formed, the white loosely compacted layer containing the majority of the synaptosomes was gently resuspended in 8 ml of 0.32 sucrose, pH 7.4. Of this synaptosomal suspension, 2 ml was placed onto 3-ml Percoll discontinuous gradients containing 0.32 M sucrose, 1 mM EDTA, 0.25 mM DL-dithiothreitol, and 3, 10, or 23% Percoll, pH 7.4. After centrifugation at 25,000 × *g* for 10 min at 4 °C, the synaptosomes were recovered from between the 10 and 23 Percoll bands and diluted in a final volume of 30 ml of HEPES buffer medium (HBM; 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4). After centrifugation at 22,000 × *g* for 10 min, the synaptosome pellet was resuspended in 8 ml of HBM medium, and the protein content was determined by the Biuret method. Finally, 1 mg of the synaptosomal suspension was diluted in 8 ml of HBM and spun at 3,000 × *g* for 10 min. The supernatant was discarded, and the pellets containing the synaptosomes were stored on ice. Under these conditions the synaptosomes remain fully viable for at least 4–6 h, as judged by the extent of KCl- and 4AP-evoked glutamate release.

Glutamate Release

Glutamate release was assayed by on-line fluorimetry as described previously (22). Synaptosomal pellets were resuspended in HBM (0.67 mg/ml) and preincubated at 37 °C for 1 h in the presence of 16 μM bovine serum albumin (BSA) to bind any free fatty acids released from synaptosomes during the preincubation. A 1-ml aliquot was transferred to a stirred cuvette containing 1 mM $NADP^+$, 50 units of glutamate dehydrogenase (Sigma) and 1.33 mM $CaCl_2$ or 200 nM free Ca^{2+}, and the fluorescence of NADPH was followed in a PerkinElmer LS-50 luminescence spectrometer at excitation and emission wavelengths of 340 and 460 nm, respectively. Traces were calibrated by the addition of 2 nmol of glutamate at the end of each assay. Data points were obtained at 2-s intervals and corrected for Ca^{2+}-independent release. Thus, the Ca^{2+}-dependent release was calculated by subtracting the release obtained during a 5-min period of depolarization at 200 nM free $[Ca^{2+}]$ from the release at 1.33 mM $CaCl_2$.

Measurements of cAMP

Synaptosomes were resuspended in incubation medium containing 16 μM BSA (2 mg/ml) and preincubated at 37 °C for 5 min. After this time, 1.33 mM $CaCl_2$ and adenosine deaminase (1 unit/mg of protein) were added followed by 1 mM 3-isobutyl-1-methylxanthine (Sigma) 10 min later, and the mix was incubated for a further 15 min. After this time, 100 μM forskolin (Roche Molecular Biochemicals) was added, and 15 min later, samples were taken. Aliquots of 0.5 ml were added to 0.15 ml of an ice-cold solution containing 1 M $HClO_4$ and 50 mM EDTA. The samples were shaken and placed on ice for 20 min. After centrifugation at 12,000 × *g* for 1 min the supernatants were neutralized with a solution of 3 M KCl and 1.5 M triethanolamine. The supernatants were collected, and cAMP content was estimated by radioimmunoassay (Amersham Biosciences).

Cytosolic Free Ca^{2+} Concentration; $[Ca^{2+}]_c$ in the Synaptosomal Population

The cytosolic free Ca^{2+} concentration was measured with fura-2. Synaptosomes were resuspended (2 mg/ml) in HBM with 16 μM BSA in the presence of 1.33 mM $CaCl_2$ and 5 μM fura-2-acetoxymethyl ester (fura 2-AM) (Molecular Probes, Eugene, OR) and incubated at 37 °C for 25 min. After fura-2 loading, synaptosomes were pelleted and resuspended in fresh HBM medium with BSA. A 1-ml aliquot was transferred to a stirred cuvette containing 1.33 mM $CaCl_2$, and the fluorescence was monitored at 340 and 510 nm. Data points were taken at 0.5-s intervals, and the cytoplasmic free Ca^{2+} concentration, $[Ca^{2+}]_c$, was calculated using the equations previously described (13).

Ca^{2+} Imaging; Ca^{2+} Responses in Single Synaptosomes

Synaptosomes in HBM (0.67 mg/ml) with 16 μM BSA were preincubated with 5 μM fura-2 AM and 1.33 mM $CaCl_2$ for 1 h. The synaptosomal suspension was attached to a polylysine-coated coverslip for another hour. A superfusion chamber was carved in a small Petri dish and mounted on a Nikon inverted stage microscope. Medium was fed into the chamber by gravity at 1–1.5 ml/min from a prewarmed (37 °C) reservoir and continuously removed by aspiration. Synaptosomes were illuminated alternately at 340 and 380 nm for 0.8 s through a 100× objective with the aid of a monochromator (Kinetic Imaging, Ltd.) and the fluorescence emitted from the nerve terminals was collected through a band-pass filter centered at 510 nm. The video images were obtained using a slow-scan CCD camera (Hamamatsu C4880) operating at 12-bit digitalization (4096 levels), and the output from the camera was stored by a computerized imaging system (Kinetic Imaging, Ltd.). The $[Ca^{2+}]_c$ was derived from the F_{340}/F_{380} ratio using the equation derived by Grynkiewicz *et al.* (23). Background images (without fura-2 fluorescence) at F_{340} and F_{380} were acquired and subtracted from each series of F_{340} and F_{380} images. $F_{340/380}$ image ratios were obtained using Kinetic Imaging software. Ratio images were stored as 32-bit floating point number data, avoiding clipping by binary digitalization. R_{max} and R_{min} parameters were determined from an *in vitro* calibration by recording fluorescence from small droplets of fura-2 (free acid, Molecular Probes) dissolved in intracellular solution (100 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 10 mM MOPS, and 10 μM fura-2, pH 7,0) plus 2 mM $CaCl_2$ (saturated Ca^{2+}) or 2 mM EGTA (0 Ca^{2+}). Individual synaptosomes were identified as bright round spots in fluorescence images. A binary mask constructed by local equalization and thresholding of row images was applied to isolate round measuring areas, zeroing pixels outside of the bright spots; in this way, some synaptosomes are not analyzed, but never more than 5–10%. For $[Ca^{2+}]_c$ measurements synaptosomes were stimulated by 10-s pulses (indicated in each graph by a bar) of 30 mM KCl in the absence and in the presence of pharmacological agonists or antagonists. Drugs were applied to the nerve terminals by switching the perfusion solution.

Immunocytochemistry for mGluR7

Antibody Staining—The affinity-purified rabbit polyclonal antibody against mGluR7 used here have been described elsewhere (24), and the monoclonal anti-synaptophysin antibody was obtained from (Sigma). To control the immunochemical reaction, primary antibodies were omitted from the staining procedure, whereupon no immunoreactivity resembling that obtained using the specific antibodies could be detected.

The synaptosomes were allowed to attach to the polylysine-coated coverslip for an hour and then fixed during 5 min in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, followed by several washes with 0.1 M phosphate buffer, pH 7.4. The synaptosomes were preincubated in 10% normal goat serum diluted in 50 mM Tris buffer, pH 7.4, containing 0.9% NaCl (TBS) with 0.2% Triton X-100 for 1 h. They were then incubated for 24 h with the affinity-purified polyclonal antibody against mGluR7 at a final protein concentration of 1–2 μg/ml and with a monoclonal antibody against synaptophysin diluted 1:1000 both in TBS containing 1% normal goat serum with 0.2% Triton X-100. After washes in TBS, the synaptosomes were incubated for 2 h in a goat anti-rabbit antibody coupled to the cyanine-derived fluorochrome Cy2 (Amersham Biosciences) and in a goat anti-mouse antibody coupled to Cy3 (Amersham Biosciences), the secondary antibodies diluted 1:200 in TBS. After several washes in TBS, the synaptosomes were cover-slipped with Fluoromount (Serva, Germany). The synaptosomes were viewed with a Nikon Diaphot microscope equipped with a 100× objective using a mercury lamp light source and fluorescein-rhodamine Nikon filter sets.

Chemicals—ω-Conotoxin-GVIA (CgTx-GVIA) and ω-agatoxin-IVA (Aga-IVA) were from Peptide Institute, Inc., Osaka, Japan. L(+)-2-amino-4-phosphonobutyrate (L-AP4). (*RS*)-α-cyclopropyl-4-phosphonophenylglycine (CPPG), and (*RS*)-α-methyl-4-phosphonophenylglycine (MPPG) were from (Tocris Cookson, Bristol, UK). H-89, calmidazolium, ophiobolin A, and staurosporine were purchased from Roche Molecular Biochemicals. Bisindolylmaleimide I, hydrochloride was from Calbiochem-Novabiochem. Sp-8-Br-cAMPS was from Biolog (Bremen, Germany). 4β-Phorbol 12,13-dibutyrate (4β-PDBu), 4α-phorbol 12,13-didecanoate (α-PDD), pertussis toxin, and $NADP^+$ were obtained from Sigma. All other reagents were from Sigma.

RESULTS

L-AP4 Reduces Glutamate Release—Depolarization of nerve terminals with both KCl and the K^+-channel blocker 4-aminopyridine (4AP) have been shown to open voltage-dependent Ca^{2+} channels and to induce the release of glutamate (25). Although both KCl and 4AP induce glutamate release to a similar extent, they use different mechanisms, as demonstrated by the insensitivity of the KCl-induced release to the Na^+-channel blocker tetrodotoxin (25). The Ca^{2+}-dependent release of glutamate after a 5-min depolarization with KCl and 4AP was 3.73 ± 0.13 (n = 17) and 3.89 ± 0.31 (n = 9) nmol of glutamate/mg ±S.E., respectively (Fig. 1, *A* and *B*). The prior

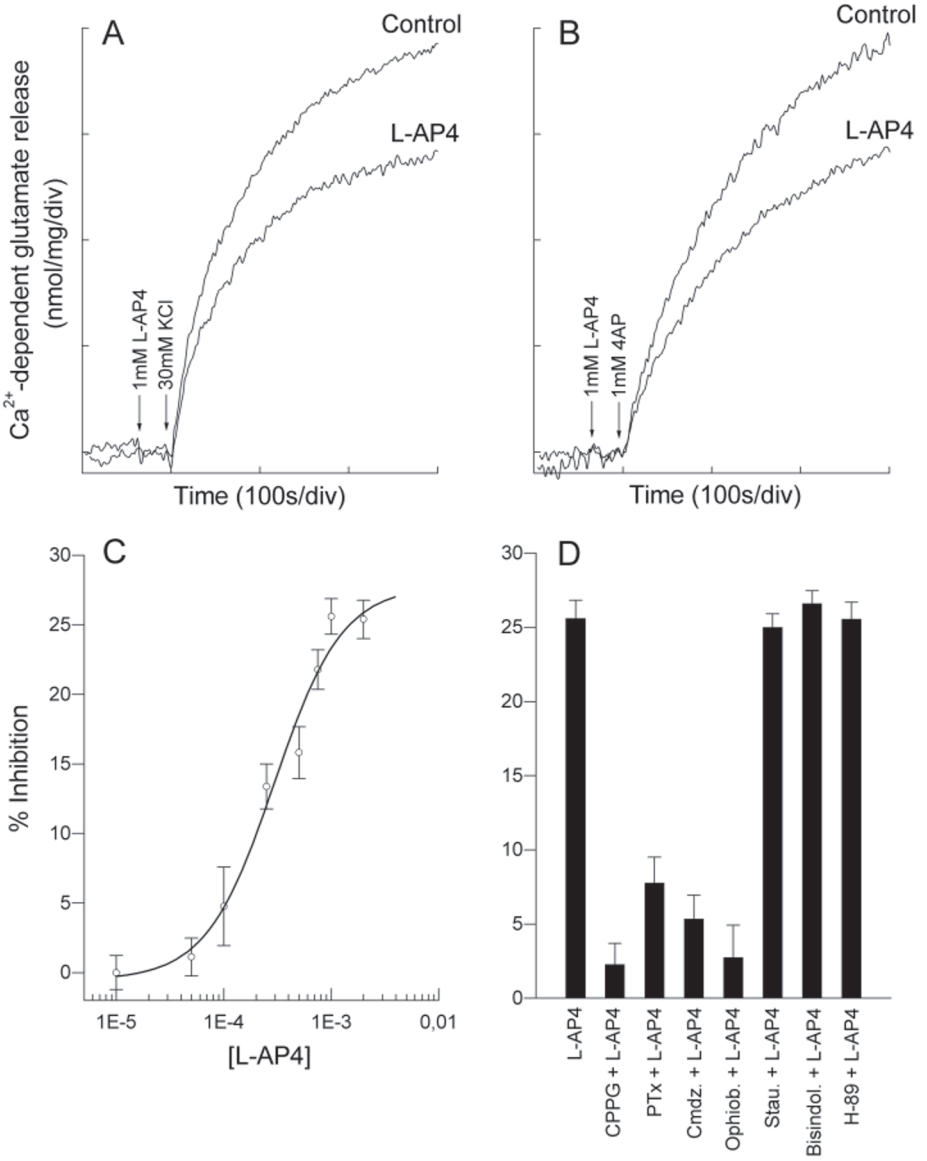

FIG. 1. **Pharmacological characterization of L-AP4 inhibition of glutamate release.** The Ca^{2+}-dependent release of glutamate was evoked by 30 mM KCl (*A*) or 1 mM 4AP (*B*) in the absence (*Control*) and in the presence of 1 mM L-AP4 added 30 s before depolarization of nerve terminals. *C*, concentration response curve of L-AP4-induced inhibition of glutamate release. Data were fitted to a sigmoid logistic model using the program Parameter Fitter (Biosoft, Cambridge, UK). *D*, percentage inhibition in the extent of Ca^{2+}-dependent release by L-AP4. The antagonist CPPG at 100 μM was added 1 min before depolarization. In the experiments with pertussis toxin (*PTx*), the synaptosomes were preincubated with the toxin (1.5 μg/ml) for 2 h. The calmodulin antagonists calmidazolium at 1 μM (*Cmdz.*) and ophiobolin at 50 μM (*Ophiob.*) and the protein kinases inhibitors staurosporine at 100 nM (*Stau.*), bisindolylmaleimide at 1 μM (*Bisindol.*), and H-89 at 10 μM were all added 30 min before depolarization. Data are the means ±S.E. values of 3–15 measurements obtained from the same number of nerve terminal preparations.

addition of the group III mGluR agonist L-AP4 (1 mM) reduced this release to 2.78 ± 0.12 (n = 15) and 2.91 ± 0.17 (n = 8) (Fig. 1, *A* and *B*). Indeed, L-AP4 inhibited the KCl-induced release in a concentration-dependent manner. The EC_{50} value for inhibition was 309.4 μM (Fig. 1*C*), indicating that the receptor reducing glutamate release in cerebrocortical nerve terminals from adult rats exhibits much lower affinity for L-AP4 than that in young rats (EC_{50} 2.3 μM) (13). Inhibition by L-AP4 was abolished by the group III mGluR antagonist CPPG (26) and was largely reduced by preincubation with pertussis toxin (*PTx*) (Fig. 1*D*), suggesting that $G_{i/o}$ proteins are involved in this presynaptic mechanism. The calmodulin antagonists calmidazolium and ophiobolin A strongly reduced presynaptic inhibition by L-AP4 (Fig. 1*D*), consistent with recent evidence of calmodulin and $\beta\gamma$ subunits binding to the intracellular carboxyl-terminal tail of group III mGluR7 in a mutually exclusive manner. Moreover, calmodulin antagonists prevent inhibition of excitatory transmission by these receptors (27). The broad spectrum protein kinases inhibitor staurosporine and the more specific PKC and PKA inhibitors bisindolylmaleimide and H-89, respectively, did not alter the inhibition of release by L-AP4, suggesting that these kinases do not participate in the signaling pathway leading to the inhibition of release (Fig. 1*D*).

Group III mGluRs reduce adenylylcyclase activity both in neuronal preparations (13, 16) as well as in heterologous expression systems (1–5). To determine whether a decrease in cAMP was responsible for the inhibition of glutamate release, we measured intrasynaptosomal cAMP levels. Basal cAMP levels (16.9 ± 2.4 pmol/mg ±S.E., n = 5) were not modified either by L-AP4 (1 mM) (16.0 ± 2.8, n = 5) or by depolarization of synaptosomes with KCl plus L-AP4 to mimic release conditions (15.0 ± 2.9 pmol/mg ±S.E., n = 5) (Fig. 2). Therefore, it seems unlikely that the L-AP4 inhibition of evoked release is related to changes in cAMP levels.

L-AP4 Reduces Ca^{2+}-Channel Activity—Another possible explanation for the inhibition of release by L-AP4-sensitive low affinity mGluRs is that the activity of Ca^{2+} channels is impaired. Given that L-AP4 reduced both KCl (tetrodotoxin-insensitive)- and 4AP (tetrodotoxin-sensitive)-evoked release, it seem more likely that the inhibitory receptors targets release-coupled Ca^{2+} channels rather than ionic channels involved in the waveform of action potentials. To better understand the mechanism of release inhibition by L-AP4, we determined the changes in the cytoplasmic free calcium concentration $[Ca^{2+}]_c$ with fura-2. The rise in $[Ca^{2+}]_c$ induced by KCl depolarization was only slightly but significantly reduced by the prior addition

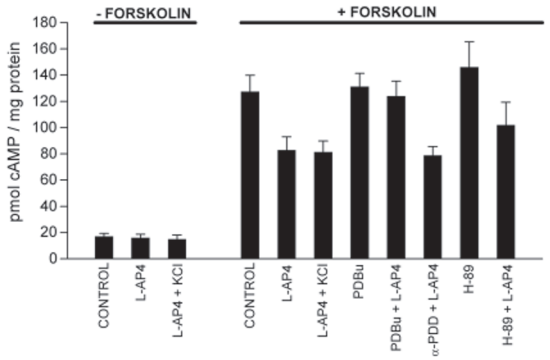

FIG. 2. **L-AP4 reduces cAMP levels in the presence but not in the absence of forskolin.** *A*, L-AP4 does not alter cAMP in the absence of forskolin. 1 mM L-AP4 alone (*L-AP4*) or in combination with 30 mM KCl (*L-AP4 + KCl*) was added to synaptosomes, and aliquots were taken 2 min later to assay cAMP levels, as described under "Experimental Procedures." Endogenous cAMP in the absence of any addition was considered as (*CONTROL*). *B*, L-AP4 reduces forskolin-stimulated cAMP levels. L-AP4 at 1 mM alone (*L-AP4*) or in combination with 30 mM KCl (*L-AP4 + KCl*) was added, and 2 min later, the samples were taken. PDBu and α-PDD, both at 1 μM, were added 1 min before the agonist L-AP4, (*PDBu + L-AP4*) and (*α-PDD + L-AP4*), respectively. In experiments with H-89, the synaptosomes were preincubated with the PKA inhibitor at 10 μM for 30 min. The results are the means ± S.E. of 3–5 experiments obtained from 3–5 preparations of synaptosomes.

of L-AP4 (Fig. 3*A*). This effect was prevented by the presence of the group II/III mGluR antagonist MPPG (28) and also by the group III mGluR antagonist CPPG (26). Activating PKC with phorbol ester PDBu before L-AP4 addition also suppressed the inhibition of release.

Glutamate release is primarily coupled to Ca^{2+} entry through both N and P/Q-type Ca^{2+}-channels (29–32), which can be selectively blocked by ω-CgTx-GVIA (33) and by ω-Aga-IVA (30), respectively. To establish which type of Ca^{2+} channel was inhibited by the L-AP4-sensitive receptor, we examined glutamate release in the presence of these Ca^{2+} channel antagonists. The KCl-evoked release was reduced by ω-Aga-IVA (200 nM) and ω-CgTx-GVIA (2 μM) by 70.8 ± 8% S.E. ($n = 3$) and 25.0 ± 3.2%, respectively (Fig. 3, *B* and *C*). The reduction induced by ω-Aga-IVA was exacerbated by the presence of L-AP4 (88.3 ± 6.7% $n = 3$), unlike that induced by ω-CgTx-GVIA (27.1 ± 2.1%, $n = 4$), indicating that a different channel population is inhibited by ω-Aga-IVA and the receptor. The preferential inhibition of *N*-type Ca^{2+} channels by L-AP4 is consistent with the non-additive effects of L-AP4 and ω-CgTx-GVIA.

PKC Suppression of the L-AP4 Inhibition of Evoked Release—The suppression of the inhibitory effects of presynaptic mGluRs mediated by PKC is a widespread phenomenon that occurs at many glutamatergic synapses (13, 34). Although the prior activation of PKC with phorbol esters completely suppressed the L-AP4 inhibition of evoked release, this suppression was not seen when protein kinase activity was inhibited with staurosporine (Fig. 4, *A* and *B*). The inactive phorbol ester α-PDD also failed to alter the L-AP4-induced reduction of glutamate release (Fig. 4*B*). PKC may mediate the suppression of the inhibitory activity of mGluRs by phosphorylation of the domains of the voltage-gated Ca^{2+}-channel that interact with the βγ subunits of the inhibitory G-proteins (35, 36). However, it has also recently been shown that PKC phosphorylates L-AP4-sensitive mGluR7 (37, 38) and that this phosphorylation suppresses receptor signaling (39).

L-AP4 Also Reduces cAMP—Activation of group III mGluRs either in neuronal preparations or in heterologous expression systems decreases cAMP levels. Indeed, in cerebrocortical nerve terminals cAMP levels were increased by forskolin (100 μM) from 16.9 ± 2.4 to 127.4 ± 12.5 pmol/mg ±S.E. ($n = 4$). The increase in cAMP induced by forskolin was significantly reduced by L-AP4 (82.9 ± 10.1 pmol/mg ±S.E., $n = 4$) (Fig. 2). Although the further addition of KCl to mimic release conditions did not alter the L-AP4-induced decrease in cAMP (81.3 ± 8.4 pmol/mg ±S.E., $n = 3$). In addition, the inhibition of PKA activity with H-89 (10 μM) slightly enhanced cAMP levels in the presence of forskolin (146 ± 19.2 pmol/mg ±S.E., $n = 3$) but did not alter the response to L-AP4 (102 ± 17.0 pmol/mg ±S.E., $n = 3$), excluding the possible negative feedback of PKA on this pathway (40). In contrast, the activation of PKC with phorbol esters (1 μM PDBu) largely suppressed the reduction in cAMP induced by L-AP4. Therefore, although the significance is unclear, in the presence of increased levels of cAMP, L-AP4-sensitive receptors decreased cAMP levels.

It is possible that the decrease in cAMP mediated by L-AP4-sensitive mGluRs serves to counteract the facilitation that results from the activation of the cAMP/PKA pathway at glutamatergic synapses (41, 42). Forskolin increases the spontaneous glutamate release through the induction of spontaneous action potentials that can be abolished by the Na^+ channel blocker tetrodotoxin (42). This forskolin-induced enhancement in the spontaneous release of glutamate (1.46 ± 0.1 nmol/mg ±S.E., $n = 3$) was largely prevented by the prior addition of L-AP4 (Fig. 5*A*), and it was also sensitive to the PKA inhibitor H-89 (Fig. 5*C*). If the L-AP4-mediated inhibition of this spontaneous release is the result of adenylylcyclase inhibition by L-AP4 and the subsequent decrease in cAMP levels, one would expect this action to be attenuated when spontaneous release is enhanced by the cAMP analogue Sp-8-Br-cAMPS, which directly activates PKA. Like forskolin, Sp-8-Br-cAMPS induced an increase in spontaneous glutamate release (1.79 ± 0.1 nmol ±S.E., $n = 3$) that was prevented by the PKA inhibitor H-89 (0.18 ± 0.1 nmol/mg ±S.E., $n = 3$). However, in contrast to that induced by forskolin, the release induced by Sp-8-Br-cAMPS was only slightly impaired by L-AP4.

To determine whether the activation of Gs-coupled receptors can also elicit the responses induced by forskolin, we determined the effect of the β-adrenergic receptor agonist isoproterenol both in cAMP levels and in glutamate release. Isoproterenol enhanced cAMP production from 18.3 ± 1.5 to 42.3 ± 2.7 pmol/mg ±S.E. ($n = 3$) (Fig. 6*A*). The increase in cAMP levels induced by isoproterenol was partially reduced by L-AP4 (30.7 ± 3.4, $n = 3$) and completely prevented by the β-adrenergic antagonist propranolol (19.7 ± 3.0, $n = 3$) (Fig. 6*A*). Isoproterenol also increased the spontaneous release of glutamate (0.56 ± 0.03 nmol/mg ±S.E., $n = 6$) (Fig. 6, *B* and *C*). This enhancement in the spontaneous release was attenuated by the prior addition of L-AP4 (0.13 ± 0.02 nmol/mg ±S.E., $n = 4$) and completely abolished by propranolol (0.01 ± 0.04 nmol/mg ±S.E., $n = 3$) (Fig. 6, *B* and *C*). These data demonstrate a functional interaction between the G_s-coupled β-adrenergic receptor and $G_{i/o}$-coupled L-AP4-sensitive mGluRs.

Calcium Imaging and Immunocytochemistry—The fact that the L-AP4 induced reduction in the Ca^{2+}-dependent release of glutamate from the population of nerve terminals was only reduced by 25% can be explained in two ways. First, although the receptor is broadly expressed in the cerebrocortical nerve terminals, it only exerts a weak effect on Ca^{2+} entry and glutamate release. Second, the expression of the L-AP4-sensitive mGluR is restricted to a fraction of nerve terminals. To distinguish between these two possibilities, Ca^{2+} imaging experiments were performed on fixed fura-2-loaded nerve terminals to determine at the single nerve terminal level to what extent the activation of L-AP4-sensitive receptors alters the influx of calcium evoked by depolarization. In a typical field of

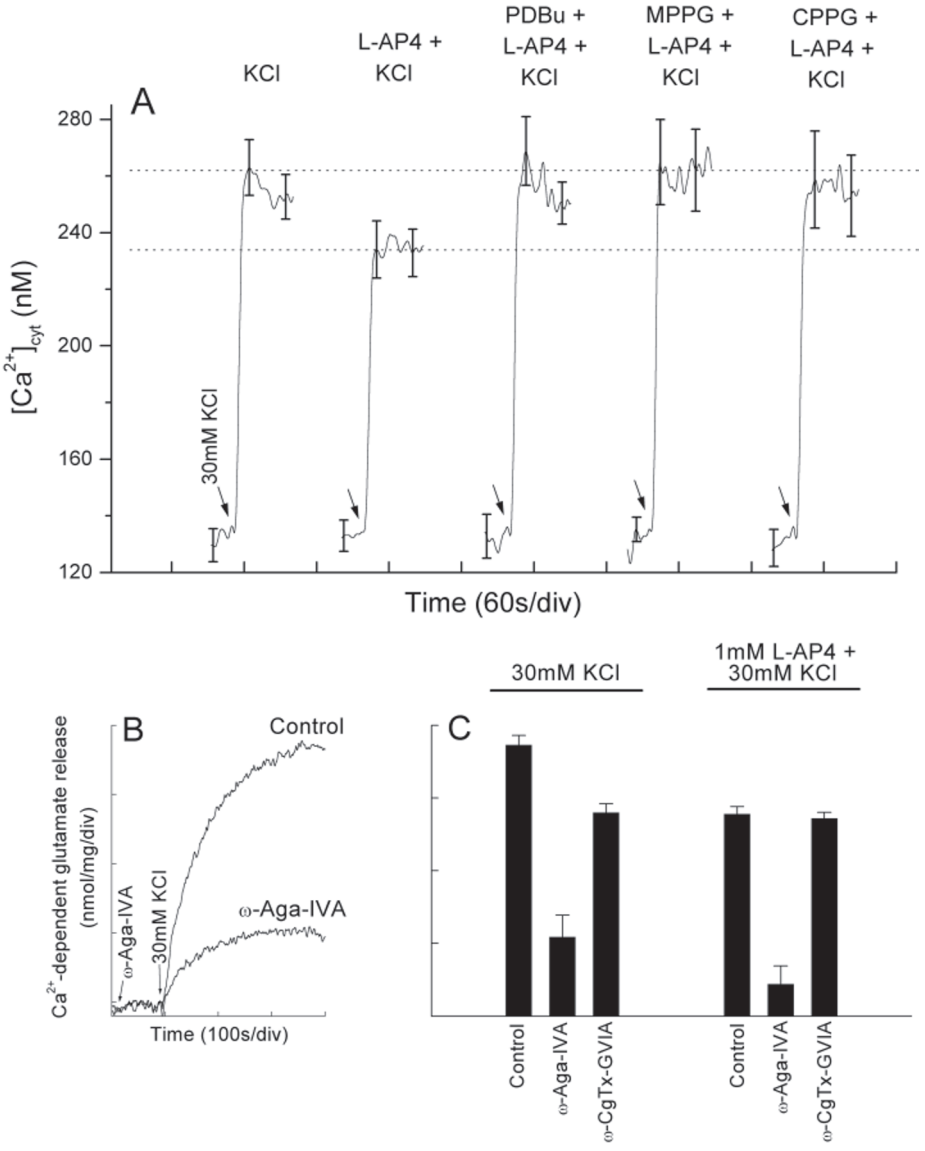

FIG. 3. **L-AP4 decreases the depolarization-evoked rise in $[Ca^{2+}]_c$ and reduces the *N*-type Ca^{2+}-channel coupled release component.** *A*, change in the KCl-induced increase in $[Ca^{2+}]_c$ in the absence (control) and in the presence of 1 mM L-AP4, which was added 30 s before KCl. The phorbol ester PDBu at 1 μM was added 10 s before L-AP4 (*PDBu + L-AP4*). The mGluR antagonists MPPG (1 mM) and CPPG (100 μM) were added 1 min before L-AP4 (*MPPG + L-AP4*) and (*CPPG + L-AP4*), respectively. *B*, Ca^{2+}-dependent release of glutamate evoked by 30 mM KCl in the absence (*Control*) and in the presence of 200 nM ω-Aga-IVA. *C*, the *bar* diagram shows the Ca^{2+}-dependent release of glutamate after 5 min of depolarization with 30 mM KCl in the absence (*Control*) and presence of the Ca^{2+} channel toxins ω-Aga-IVA at 200 nM or ω-CTx-GVIA at 2 μM, both in the absence and in the presence of 1 mM L-AP4. The results are the means ± S.E. of 3–5 experiments obtained from the same number of synaptosomal preparations.

synaptosomes, individual synaptosomes were abundant, but they were also observed within clusters. In size, they ranged from 0.5 to 1.2 μm in diameter, they were typically circular and displayed strong fluorescence when loaded with fura-2, and autofluorescence was not significant (Fig. 7*A*). Among these fura-2-loaded synaptosomes more than 95% responded to 30 mM KCl, and the basal $[Ca^{2+}]_c$ was around 119 ± 30 nM. Although heterogeneous in amplitude, ranging from 500 to 900 nM, the response was always transient, in contrast to the more sustained Ca^{2+} responses observed with prolonged depolarizations in individual nerve terminals (43). A total of 4,191 fura-2-loaded particles responding to KCl and obtained from 7 fields similar to that shown in Fig. 7*A* were analyzed. Among this population, 28.4% ± 2.2 (1,188 of 4,191) were responsive to L-AP4 (1 mM), whereas the rest (71.6 ± 2.2%) were not. The average Ca^{2+} response of individual nerve terminals to a 10-s application of 30 mM KCl was transient and strongly reduced by the co-application of 30 mM KCl and 1 mM L-AP4 (Fig. 7*B*). The effects by L-AP4 were reversible, as a further addition of 30 mM KCl restored the Ca^{2+} response. The depolarization of nerve terminals with KCl in the absence and presence of L-AP4 produced similar Ca^{2+} responses in nerve terminals unresponsive to 1 mM L-AP4 (Fig. 7*C*). The L-AP4-induced inhibition of Ca^{2+} influx was completely reversed by the group III mGluR antagonist CPPG (Fig. 8*A*). The KCl-induced Ca^{2+} response was not altered by low concentrations of L-AP4 (10 μM) in any of the nerve terminals tested (1,008) (Fig. 8*B*). Thus, in contrast to nerve terminal preparations from young rats (13), cerebrocortical nerve terminals from adult (2–3 months) rats do not contain mGluRs with high affinity for L-AP4.

The fact that the L-AP4-sensitive mGluR that inhibits glutamate release has a low affinity for the agonist prompted us to analyze the distribution of mGluR7 by immunochemistry. To this end, synaptosomes were fixed onto polylysine-coated coverslips and double-labeled with an antibody against the vesicular marker protein synaptophysin and with an antisera against mGluR7. In these experiments fields with fewer nerve terminals were used to facilitate isolation. Among the particles that contained synaptophysin (Fig. 9*A*) (230 in 10 fields) mGluR7 was detected in 25–35% (Fig. 9*B*). The merged field is shown in Fig. 9*C*.

DISCUSSION

In this paper we show that the inhibition of glutamate release by high (1 mM) concentrations of L-AP4 in cerebrocortical nerve terminals from adult rats is mediated by mGluR7. Two

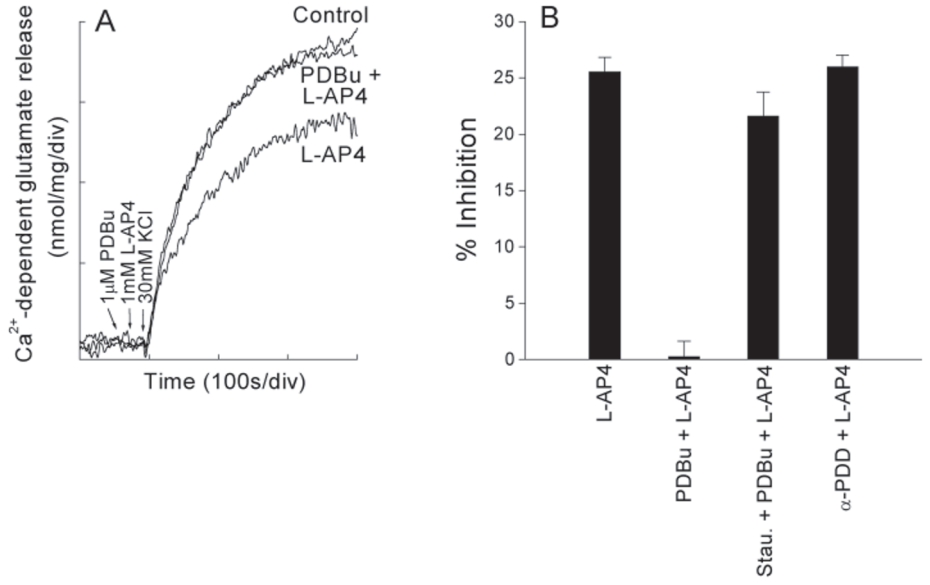

FIG. 4. **PKC-mediated suppression of the L-AP4-induced inhibition of glutamate release.** *A*, the glutamate release evoked by 30 mM KCl (*Control*) was reduced by 1 mM L-AP4 added 30 s before depolarization (*L-AP4*). The phorbol ester PDBu (1 μM) was added 10 s before L-AP4 (*PDBu + L-AP4*). *B*, the *bar* diagram shows the Ca^{2+}-dependent release of glutamate after 5 min of depolarization with 30 mM KCl. The suppression by PDBu of the L-AP4-induced inhibition was also determined in synaptosomes incubated with staurosporine (100 nM, 30 min) (*Stau. + PDBu + L-AP4*). The inactive phorbol ester α-PDD at 1 μM was added 10 s before L-AP4 (*α-PDD + L-AP4*). The results are the means ± S.E. of (3–7) experiments obtained from 3–7 preparations of synaptosomes.

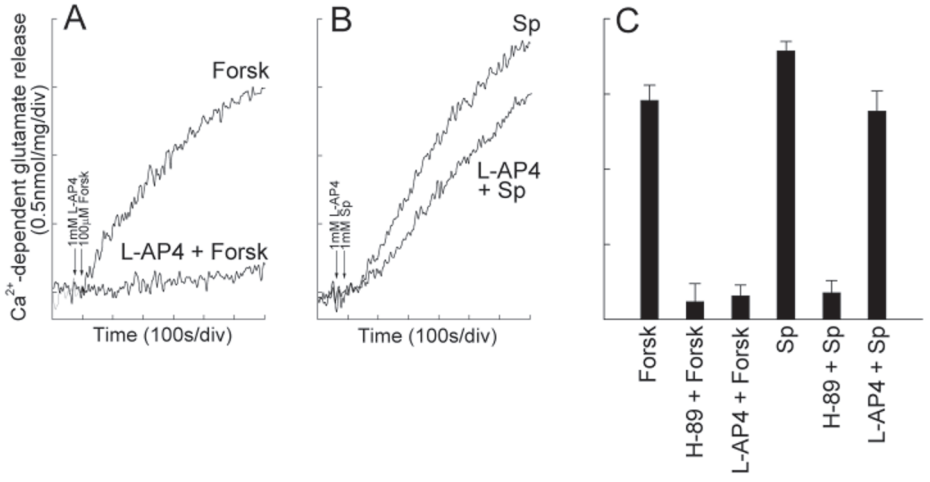

FIG. 5. **L-AP4 signaling through a decrease in cAMP is important to antagonize the forskolin-induced increase in spontaneous release.** *A*, the spontaneous Ca^{2+}-dependent release induced by 100 μM forskolin (*Forsk*) was antagonized by 1 mM L-AP4 (*L-AP4 + Forsk*) added 10 s before the adenylyl cyclase activator. *B*, the release induced by the cAMP analogue Sp-8-Br-cAMPS at 1 mM (*Sp*) was only slightly impaired by 1 mM L-AP4 (*L-AP4 + Sp*). *C*, *bar* diagrams show the cumulative Ca^{2+}-dependent release (mean ± S.D., $n = 3–4$) induced by 100 μM forskolin or 1 mM Sp-8-Br-cAMPS over 10 min. In experiments with H-89, the synaptosomes were preincubated with the PKA inhibitor (10 μM) for 30 min (*H-89 + Forsk*) and (*H-89 + Sp*). The results are the means ± S.E. of 3–4 experiments obtained from 3–4 preparations of synaptosomes.

signaling pathways are involved in this process. The inhibition of evoked release correlated with a reduction in the activity of N type Ca^{2+} channels without any detectable change in cAMP levels. However, the enhancement of cAMP levels with forskolin uncovered the ability of the receptor to modulate this signaling pathway by reducing the levels of cAMP, thereby counteracting the facilitation of glutamate release that results from PKA activation. The global reduction of the Ca^{2+}-dependent release of glutamate induced by L-AP4 only reaches 25% of the total release observed. This is due to the expression of mGluR7 being restricted to a sub-population of the nerve terminals rather than to the low efficiency of the presynaptic mechanisms, which in fact dramatically reduced both Ca^{2+} entry and glutamate release in mGluR7-expressing nerve terminals.

mGluR7 Mediates Release Inhibition by 1 mM L-AP4—Immunolabeling with mGluR7 antibodies (9, 24, 44, 45) together with Ca^{2+} imaging of single nerve terminals was performed to examine the $[Ca^{2+}]_c$ in mGluR7-expressing nerve terminals. Together, these techniques revealed that the population of mGluR7-expressing nerve terminals largely responded to L-AP4 by reducing their $[Ca^{2+}]_c$, indicating that mGluR7 is the principal receptor involved in the reduction of glutamate release at glutamatergic nerve terminals in the cerebral cortex from adult rats. This is consistent with high levels of expression of both mGluR7 mRNA (3, 4, 46, 47) and protein (8, 48) in the cerebral cortex of adult rats. The high concentration of L-AP4 required to inhibit release in cerebrocortical nerve terminals is also in agreement with the low affinities of mGluR7 for L-AP4 and glutamate observed in heterologous systems (3, 4, 47). Although other group III metabotropic glutamate recep-

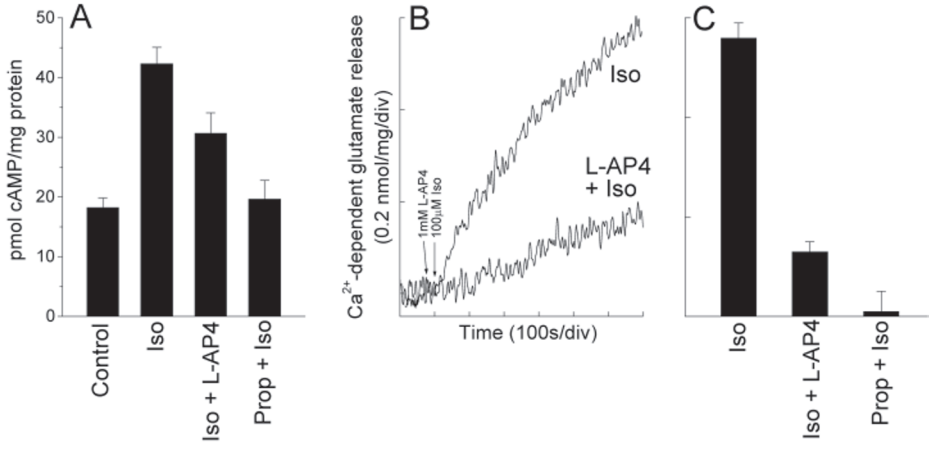

FIG. 6. **L-AP4 reduces the cAMP levels and the glutamate release induced by the activation of β-adrenergic receptors.** *A*, L-AP4 reduces isoproterenol-stimulated cAMP levels. Endogenous cAMP in the absence of any addition was considered as control. Isoproterenol (*Iso*) at 100 μM was present for 15 min. L-AP4 at 100 μM was added 13 min after isoproterenol (*Iso + L-AP4*). The β-adrenergic antagonist propranolol (100 μM) was added 1 min before isoproterenol (*Prop + Iso*). *B*, the spontaneous release of glutamate induced by 100 μM isoproterenol was reduced by 1 mM L-AP4 (*L-AP4 + Iso*) added 10 s before the β-adrenergic agonist. *C*, *bar* diagrams show the cumulative Ca^{2+}-dependent release induced by 100 μM isoproterenol in the absence (*Iso*) or in the presence of L-AP4 (*Iso + L-AP4*) or propranolol (*Prop + Iso*) over 10 min. The results are the means ± S.E. of 3–6 experiments obtained from 3–6 preparations of synaptosomes.

tors such as mGluR4 and mGluR8 are also expressed in the brain, the expression of mGluR4 is weaker in the cortex than that of mGluR7, and mGluR8 expression is restricted to the piriform cortex (49). In addition, mGluR4 and mGluR8 both show high affinity for L-AP4 in modulating G-protein-coupled effectors such as adenyl cyclase (5) or inwardly rectifying potassium channels (50). In cerebrocortical nerve terminals from adult rats, the inhibition of glutamate release by high affinity L-AP4-sensitive mGluRs was virtually absent. A reduction of the Ca^{2+} responses in these nerve terminals in the presence of 10 μM L-AP4 is only observed in 0.2% of the nerve terminals (see Fig. 8*B*).

During development, a switch occurs in the modulation of glutamate release by group III mGluRs. In contrast to adult rats (2–3 months), high affinity L-AP4-sensitive mGluRs reduced glutamate release in cerebrocortical nerve terminals from young rats (2–3 weeks) (18). Meanwhile, adult rats express low affinity L-AP4-sensitive mGluRs that modulate the inhibition of release. This developmentally controlled switch has also been observed in the CA1 area of the hippocampus, where a dramatic decrease in the control of synaptic transmission by high affinity L-AP4-sensitive mGluRs occurs during development (17). Nevertheless, high concentrations of L-AP4 strongly depressed synaptic transmission in older rats (12, 39).

Immunohistochemical localization of synaptophysin in different brain areas has shown that only 7% of the synaptophysin-labeled particles could not be positively identified as nerve terminal-containing vesicles, whereas 93–99% of vesiculated axon profiles contained synaptophysin (51). As such, we can assume that synaptophysin is a reliable marker for axon terminals. Double immuno-labeling indicates that in the synaptosomal preparation, 25–35% of the synaptophysin-positive particles also contain mGluR7. Similar results were obtained with gold-conjugated antibodies against mGluR7, where 25–33% of the nerve terminals positively identified as vesicle-containing axon profiles were labeled for mGluR7 (data not shown). These data correlate with that obtained from Ca^{2+}-imaging experiments, where 28% of the nerve terminals responded to 1 mM L-AP4. Although immunolabeling studies and Ca^{2+}-imaging experiments will detect mGluR7 in nerve terminals other than glutamatergic ones, the fact that L-AP4 1 mM decreases the Ca^{2+}-dependent release of glutamate by 25% suggests that the majority of the nerve terminals containing mGluR7 are glutamatergic. Thus, although mGluR7 is also present at GABAergic nerve terminals, this represents a small subpopulation of nerve terminals (52).

mGluR7 Inhibits N-type Ca^{2+} Channels—In cerebrocortical nerve terminals L-AP4 reduced the depolarization-evoked rise in $[Ca^{2+}]_c$ and glutamate release, consistent with previous findings where voltage-dependent Ca^{2+} channels where inhibited by group III mGluRs (11, 14, 15, 32, 53). Glutamate release in nerve terminals is coupled to Ca^{2+} entry through both N and P/Q types of calcium channels (32). However, it appears that mGluR7 inhibits *N*-type calcium channels since an additive effect was observed with ω-agatoxin IVA but not with the *N*-type channel blocker ω-conotoxin GVIA. The inhibition of the evoked release by mGluR7 occurs without any detectable change in cAMP levels. In addition, this inhibitory action was insensitive to PKA and PKC inhibitors, suggesting a membrane-delimited interaction between $G_{i/o}$-protein βγ subunits and the N channel in the absence of any intracellular messenger active on PKA or PKC. This mechanism of release inhibition is consistent with the localization of mGluR7 to presynaptic active zones of asymmetrical synapses (9, 44, 45). Somatic mGluR7 has been shown to inhibit the activity of P/Q type Ca^{2+} channels in transfected cerebellar cells (53). However, this signaling involves phospholipase C and PKC activation and clearly differs from that used by mGluR7 in nerve terminals.

L-AP4 inhibits the Ca^{2+}-dependent release of glutamate from the whole cerebrocortical nerve terminals population by 25%, whereas a slightly larger fraction of these nerve terminals express mGluR7 (25–35%). These data clearly suggest that this receptor, although restricted to a fraction of nerve terminals, exerts a strong inhibitory effect on glutamate release. Indeed, Ca^{2+} imaging of individual nerve terminals showed that L-AP4 induced a dramatic inhibition of Ca^{2+} entry. Thus, given the high local $[Ca^{2+}]_c$ that triggers release at the active zone (54) it is very likely that the reduction in Ca^{2+} entry after mGluR7 activation prevents the firing of the mGluR7-containing nerve terminals during KCl stimulation. Therefore, it can be assumed that the 25% reduction in the total Ca^{2+}-dependent release of the synaptosomal preparation induced by L-AP4 is the result of the complete inhibition of release from the subpopulation of mGluR7-containing nerve terminals. This is consistent with the strong inhibition of synaptic transmission by L-AP4 found at some glutamatergic synapses (12, 39).

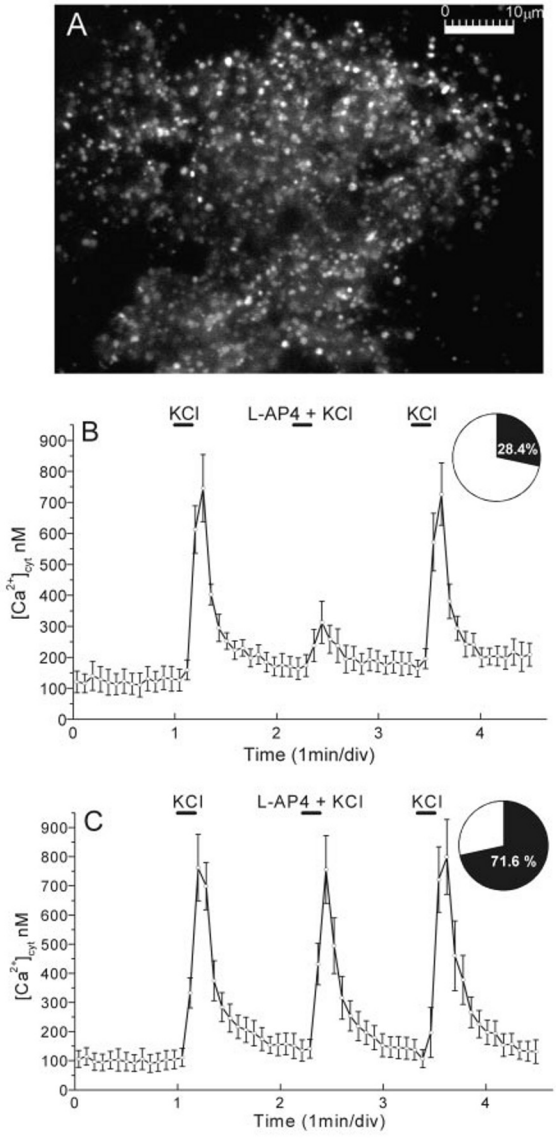

FIG. 7. **L-AP4 largely suppresses the Ca^{2+} responses by KCl in individual nerve terminals.** Synaptosomes were fixed onto polylysine-coated coverslips and loaded with fura-2 as indicated under "Materials and Methods." *A*, representative field of fura-2-loaded synaptosomes under basal conditions. Ca^{2+} responses were induced by a 10-s application of 30 mM KCl in the absence (*KCl*) and in the presence of 1 mM L-AP4 (*L-AP4 + KCl*). Data are the means ± S.E. of 8–10 responses of individual nerve terminals. The disc diagram inserted in *black* indicates the % of nerve terminals showing a given response.

Signaling through mGluR7 to inhibit glutamate release does not involve PKC or PKA activation since inhibitors of these kinases did not affect release inhibition. However, activation of PKC disrupts coupling between the receptor and G-proteins, preventing the decreases in cAMP, $[Ca^{2+}]_c$, and glutamate release induced by L-AP4. This finding is consistent with previous data demonstrating that PKC disrupt the L-AP4 inhibition of Ca^{2+} channels, synaptic transmission, and glutamate release (13, 34). More recently it has been shown that PKC phosphorylates mGluR7 (37) and disrupts the signaling of group III mGluRs (39). It is also possible that the PKC-mediated suppression of the inhibitory response of L-AP4 in glutamate release results from the interaction of the PKC-signaling pathway downstream of the G-protein. This could result from the phosphorylation of the Ca^{2+} channel and the suppression of the inhibitory action of the $\beta\gamma$ subunits of $G_{i/o}$ on Ca^{2+} channel activity (36, 55).

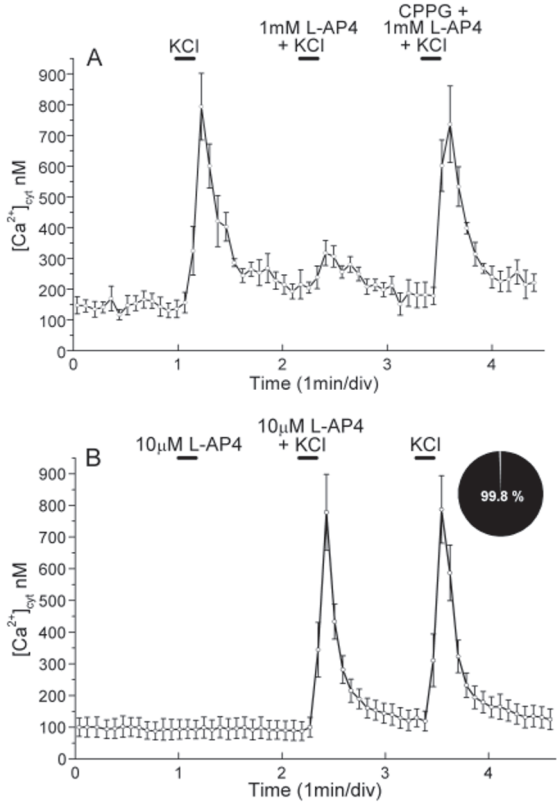

FIG. 8. **CPPG antagonizes the effects of L-AP4 in Ca^{2+} responses.** *A*, Ca^{2+} responses were induced by a 10-s application of 30 mM KCl in the absence (*KCl*) and in the presence of 1 mM L-AP4 (*L-AP4 + KCl*). The decrease in Ca^{2+} responses by L-AP4 was abolished by the mGluR antagonist CPPG at 100 μM (*CPPG + L-AP4 + KCl*). Low concentrations of L-AP4 (10 μM) did not alter the Ca^{2+} responses to KCl (*B*). Data are the means ± S.E. of 8–10 responses by individual nerve terminals. Inserted disc diagrams in *black* indicate the % of nerve terminals showing a given response.

L-AP4 Also Decreases cAMP—Although group III mGluRs inhibit adenyl cyclase in heterologous expression systems (3, 4), the physiological relevance of this signaling at nerve terminals remains unclear. In this paper we discovered the ability of mGluR7 to reduce cAMP levels after an increase in intrasynaptosomal cAMP concentrations. Moreover, this signaling mechanism antagonizes the increase in the spontaneous release of glutamate induced by the cAMP/PKA-mediated pathway. Thus, L-AP4 efficiently antagonized the forskolin-induced release but not the release induced by cAMP analogues that acts downstream of adenyl cyclase at the level of PKA. It might be argued that the inhibition of spontaneous release results from a reduction in Ca^{2+} channel activity. However, if this inhibitory mechanism of release were still active under conditions of PKA activation, it would be expected to affect both forskolin- and Sp-8-Br-cAMPS-induced release equally. Although the precise physiological role of signaling through a decrease in cAMP levels is not entirely clear, our data suggest that signaling through this pathway provides a way to balance the potentiation of transmission at glutamatergic synapses induced by the increase in cAMP and PKA activation.

The presence in the cortex of a presynaptic β-adrenergic receptor linked to the G_s protein and adenylyl cyclase/PKA activation is consistent with the evidence that noradrenergic neurons from the locus ceruleus innervate the cerebral cortex (56). Although the β-adrenergic agonist mimics the action of forskolin to increase both cAMP and the spontaneous release of glutamate, this occurs to a lesser extent, suggesting that other presynaptic receptors can also be coupled to the G_s/adenylyl

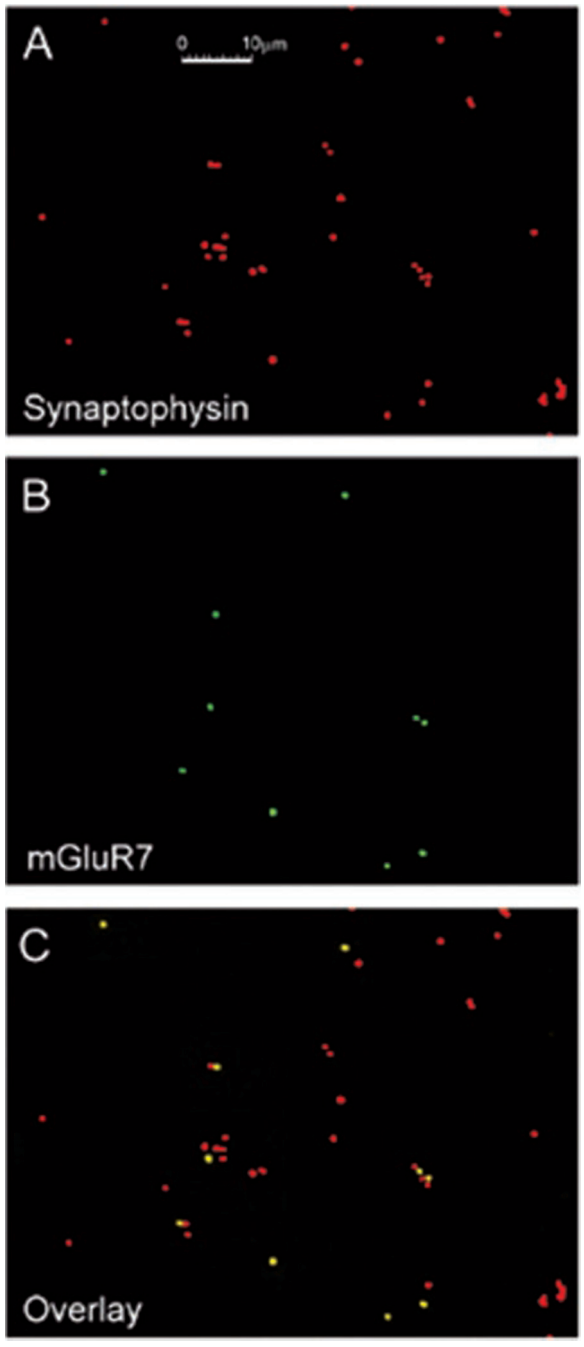

FIG. 9. **mGluR7 mediates the L-AP4 reduction in Ca^{2+} responses.** Synaptosomes were fixed onto polylysine-coated coverslips, and images of doubly stained nerve terminals were obtained. Nerve terminals were visualized with Cy3 optics for synaptophysin (*A*) and with Cy2 optics for mGluR7 (*B*). *C*, merged *panels A* and *B*.

cyclase/PKA pathway in this region. Interestingly, the facilitatory action of isoproterenol was antagonized by L-AP4, suggesting the coexistence in nerve terminals of receptors coupled to G_s and G_i proteins and, therefore, involved in the increase and in the reduction of cAMP levels, respectively. A functional interaction between L-AP4-sensitive mGluRs and β-adrenergic receptors in the control of glutamate release has recently been observed in hippocampal synapses (40).

This paper provides evidence for the double signaling of mGluR7 through a decrease in Ca^{2+} and cAMP to inhibit release. Considering this alongside the requirement of calmodulin binding for release inhibition (27) and the suppression of release inhibition by PKC, it is suggested that mGluR7 expressing nerve terminals integrate signals mediated by receptors others than mGluR7 at the level of second messengers (Ca^{2+} and cAMP) and protein kinase activity.

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