Novel glial–neuronal signalling by coactivation of metabotropic glutamate and β-adrenergic receptors in rat hippocampus

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1. We have previously reported that activation of group II-like metabotropic glutamate receptors (mGluRs) in rat hippocampus results in a potentiation of the accumulation of cAMP elicited by activation of G-protein G_s-coupled receptors. This large increase in cAMP levels results in release of cAMP or a cAMP metabolite and depression of synaptic transmission at the Schaffer collateral–CA1 pyramidal cell synapse through activation of A_1 adenosine receptors.

2. Consistent with these studies, we report that antagonists of group II mGluRs block both the potentiation of cAMP accumulation elicited by activation of mGluRs and the depression of synaptic transmission induced by coactivation of mGluRs and β-adrenergic receptors.

3. *In situ* hybridization studies suggest that of the cloned group II mGluRs only mGluR-3 mRNA is present in area CA1. Interestingly, mGluR-3 appears to be present predominantly in glia in this region. Thus, we tested the hypothesis that mGluRs coupled to potentiation of cAMP accumulation were present on glia rather than neurons in area CA1.

4. The selective group II mGluR agonist 2*S*,1′*R*,2′*R*,3′*R*-2-(2,3-dicarboxycyclo-propyl)glycine (DCG-IV) failed to enhance cAMP-mediated electrophysiological responses to the β-adrenergic receptor agonist isoprenaline (Iso) in CA1 pyramidal cells, suggesting that mGluRs coupled to potentiation of cAMP accumulation may not be present in these cells.

5. Pre-incubation of hippocampal slices with either of the selective glial toxins L-α-amino-adipic acid (L-AA) or fluorocitrate (FC) blocked mGluR-mediated potentiation of cAMP accumulation. However, L-AA and FC had no discernible effects on viability of CA1 pyramidal cells, or cAMP-mediated electrophysiological effects in these neurons.

6. Pre-incubation of hippocampal slices with the neurotoxin kainate resulted in disruption of neuronal transmission and degeneration of neurons in area CA1, but had no effect on mGluR-mediated potentiation of cAMP accumulation.

7. Pre-incubation of hippocampal slices with the cAMP/cAMP metabolite transport blocker probenicid blocked the depression of synaptic transmission elicited by coapplication of Iso and DCG-IV, while having no significant effect on cAMP accumulation elicited by these agonists.

8. Taken together, these data suggest that mGluRs coupled to potentiation of cAMP accumulation are present on glia rather than neurons in area CA1 of hippocampus. This suggests that a novel form of glial–neuronal communication may exist, since activation of these mGluRs in concert with β-adrenergic receptors results in depression of synaptic transmission.

Glutamate, the primary excitatory neurotransmitter in the mammalian CNS, elicits its actions by activation of both ionotropic and metabotropic glutamate receptors (mGluRs). Ionotropic glutamate receptors are ligand-gated cation channels that mediate fast synaptic transmission. In contrast, mGluRs are G-protein-coupled receptors that regulate various second messenger systems. Since the discovery of the first mGluR subtype (mGluR-1) by

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expression cloning in *Xenopus* oocytes, a large family of at least eight mGluR subtypes (designated mGluR-1 to mGluR-8) has been cloned and sub-categorized into three groups based on amino acid sequence homology and pharmacological profile (for review see Pin & Duvoisin, 1995; Gereau & Conn, 1995*a*). Group I consists of mGluR-1 and mGluR-5, which are selectively activated by 3,5-dihydroxyphenylglycine (DHPG). Group II includes mGluR-2 and mGluR-3, which are selectively activated by 2*S*,1′*R*,2′*R*,3′*R*-2-(2,3-dicarboxycyclopropyl)-glycine (DCG-IV). Group III mGluRs are selectively activated by L-2-amino-4-phosphonobutyric acid (L-AP4), and include mGluR-4, mGluR-6, mGluR-7 and mGluR-8.

Activation of mGluRs by the non-selective mGluR agonist 1-aminocyclopentane-1*S*,3*R*-dicarboxylic acid (1*S*,3*R*-ACPD) leads to modulation of a variety of second messenger systems in rat hippocampus, including stimulation of phosphoinositide hydrolysis, inhibition of forskolin-stimulated cAMP accumulation, and activation of phospholipase D (for review see Pin & Duvoisin, 1995). In addition, we and others have reported that activation of mGluRs in hippocampal slices can also increase cAMP formation (Casabona, Genazzani, Di Stefano, Sortino & Nicoletti 1992; Winder & Conn, 1992; Schoepp & Johnson, 1993). This increase in cAMP accumulation is mediated by activation of mGluRs in hippocampus that potentiate the accumulation of cAMP elicited by activation of other receptors that are directly coupled to adenylyl cyclase through the G-protein G_s, such as β-adrenergic (βAR), vasoactive intestinal polypeptide (VIP), prostaglandin E_2 (PGE_2) and A_2 adenosine receptors (Genazzani *et al.* 1993; Schoepp & Johnson, 1993; Winder & Conn, 1993). Activation of mGluRs does slightly increase basal cAMP accumulation in hippocampus. However, this is mediated by potentiation of the cAMP accumulation elicited by endogenous extracellular adenosine acting at A_2 receptors, rather than coupling of an mGluR to adenylyl cyclase through G_s (Winder & Conn, 1993). Thus, an mGluR exists in hippocampus that belongs to a growing family of receptors, including α-adrenergic, $GABA_B$ and others, that potentiate cAMP formation elicited by activation of G_s-coupled receptors.

Metabotropic glutamate receptor-induced potentiation of G_s-mediated increases in cAMP accumulation is an interesting effector system in that it provides a coincidence detector that allows for associative modulation of the levels of cAMP. It has been hypothesized that such coincidence detection by adenylyl cyclase could play an important role in neuronal signalling (Anholt, 1994). Consistent with this hypothesis, coactivation of mGluRs and βARs, but neither alone, results in a novel associative depression of transmission at the Schaffer collateral–CA1 pyramidal cell (SC–CA1) synapse in adult rat hippocampus (Gereau & Conn, 1994*a*, *b*; Gereau, Winder & Conn, 1995). A number of studies strongly suggest that this depression of excitatory transmission is mediated by the synergistic increase in cAMP accumulation elicited by coapplication of mGluR and βAR agonists. For instance, the depression of transmission can only be elicited by coapplication of agonists of βARs with agonists of the specific mGluRs that potentiate cAMP responses (Gereau & Conn, 1994*b*; Gereau *et al.* 1995). Second, L-serine-*O*-phosphate completely inhibits the synergistic increase in cAMP accumulation and inhibits the depression of transmission elicited by activation of mGluRs and βARs (Gereau & Conn, 1994*b*; Winder, Smith & Conn, 1993). Third, the depression of transmission induced by coactivation of mGluRs and βARs is mimicked by application of the adenylyl cyclase activator forskolin, or application of cAMP analogues (Dunwiddie, Taylor, Heginbotham & Proctor, 1992). Finally, depression of excitatory synaptic transmission by coapplication of the β-adrenergic receptor agonist isoprenaline (Iso) and 1*S*,3*S*-ACPD is blocked by SQ 22536, an inhibitor of adenylyl cyclase (Gereau *et al.* 1994*b*). Taken together, these data strongly suggest that the depression of excitatory synaptic transmission elicited by coactivation of mGluRs and βARs is mediated by the large synergistic increase in cAMP accumulation. However, cAMP-mediated depression of EPSCs is unique in that it is not dependent on activation of cAMP-dependent protein kinase (Gereau & Conn, 1994*b*). Rather, the depression of excitatory transmission is mediated by a novel protein kinase-independent mechanism in which cAMP metabolites are released into the extracellular space where they activate presynaptic A_1 adenosine receptors on Schaffer collateral terminals (Gereau & Conn, 1994*b*).

Extensive pharmacological analysis of the mGluR coupled to both potentiation of cAMP accumulation and to depression of synaptic transmission when coactivated with βARs suggests that the receptor that mediates these responses is a group II mGluR (mGluR-2 and/or mGluR-3; Winder & Conn, 1993; Winder *et al.* 1993; Gereau & Conn, 1994; Gereau *et al.* 1995; Winder & Conn, 1995). *In situ* hybridization reveals that of the cloned group II mGluRs, only mGluR-3 mRNA is present in area CA1 (for review see Testa, Catania & Young, 1994). Interestingly, while mGluR-3 mRNA is present in area CA1, it appears to be predominantly localized in glia rather than neurons (for review see Testa *et al.* 1994). These data raise the possibility that mGluRs coupled to potentiation of cAMP accumulation may be localized on glia within the hippocampus rather than on neurons. Consistent with this hypothesis, Schwartz (1993) has reported that an mGluR in Müller glia from salamander retina conditionally modulates a K^+ current by potentiating the effects of cAMP. It is interesting to note that, because of the novel mechanism for the depression of transmission elicited by coactivation of βARs and mGluRs in hippocampus, the receptors responsible for eliciting the cAMP response need not be present on neurons. This is an exciting possibility since it would suggest that coactivation of these receptors located on glia could acutely regulate hippocampal synaptic transmission. Thus, we performed

experiments aimed at testing the hypothesis that mGluRs coupled to potentiation of cAMP in area CA1 of hippocampus are predominantly localized on glia.

METHODS

Experimental procedures

For all experiments, unanaesthetized male Sprague–Dawley rats were rapidly decapitated, the brain removed, and the hippocampus dissected on ice. All procedures were reviewed and approved by the Emory University Animal Care and Use Committee.

Measurement of cAMP Levels

Increases in cAMP accumulation were measured as previously described (Winder & Conn, 1992). This method involves measurement of agonist-induced accumulation of [^{3}H]cyclic AMP in rat brain slices prelabelled with [^{3}H]adenine. For experiments involving pretreatment with fluorocitrate (FC) or L-α-aminoadipic acid (L-AA), cross-chopped hippocampal slices (350 μm × 350 μm) from male Sprague–Dawley rats (100–175 g) were prepared and incubated in oxygenated Krebs–bicarbonate buffer (KRB; composition (mM): 108 NaCl, 4·7 KCl, 2·5 $CaCl_2$, 1·2 $MgSO_4$, 1·2 KH_2PO_4, 10 glucose, 25 $NaHCO_3$) for 15 min. Cross-chopped hippocampal slices were prepared as previously described (Winder & Conn, 1993). After a 15 min incubation period in warmed KRB, the tissue was then incubated for 40 min in 15 ml KRB containing 30 μCi [^{3}H]adenine (American Radiolabelled Chemicals, St Louis, MO, USA) and 6 μM unlabelled adenine. After several rinses with warm KRB, 25 μl of gravity-packed slices were transferred to each incubation tube and incubated for varying times with FC, L-AA, or KRB, after which agonists were added to the vials and then incubated for a further 15 min (final volume, 0·5 ml). For experiments involving pre-incubation with kainate, 400 μm-thick transverse hippocampal slices were prepared from male Sprague–Dawley rats (125–150 g) and incubated in oxygenated KRB, at 37 °C for 30 min. Slices were then incubated in 100 μM kainate or KRB for the appropriate time, after which the tissue was washed and incubated for 40 min in 15 ml KRB containing 30 μCi [^{3}H]adenine (American Radiolabelled Chemicals) and 6 μM unlabelled adenine. After several rinses with warm KRB, two slices were transferred to each incubation tube and incubated for 15 min with appropriate drugs (final volume, 0·5 ml). In both cases, the reaction was terminated with 50 μl of 77% trichloroacetic acid, and 25 μl of 10 mM cyclic AMP was added as a carrier. The tissue was homogenized, centrifuged (15 min at 37 000 *g*) and 25 μl of the supernatant was removed for determination of total radioactivity incorporated into the tissue. [^{3}H]Cyclic AMP in the remaining supernatant was isolated by sequential elution through Dowex (Sigma) and then alumina columns. The results were expressed as percent conversion of total radioactivity to [^{3}H]cyclic AMP. All incubations were at 37 °C under an atmosphere of 95% O_2–5% CO_2 in a shaking water bath.

Electrophysiology

Transverse hippocampal slices were prepared for electrophysiological studies as previously described (Gereau & Conn, 1994*a*, *b*). The hippocampus was rapidly unilaterally dissected out on ice, and 400 μm slices were cut on a McIlwain tissue chopper and placed in oxygenated artificial cerebrospinal fluid (ACSF; composition (mM): 124 NaCl, 2·5 KCl, 2 $CaCl_2$, 1·3 $MgSO_4$, 1 NaH_2PO_4, 10 glucose, 26 $NaHCO_3$) at room temperature (23–26 °C) for 1 h. After equilibration, a slice was taken from the holding chamber and placed in a standard submerged brain slice recording chamber perfused with oxygenated ACSF (1 ml min^{-1}) at 30 °C for intracellular and extracellular recordings, or at room temperature for whole-cell patch clamp experiments.

Whole-cell patch clamp recordings were obtained with either a List EPC-7 or Warner Instruments Corp. (Hamden, CT, USA) PC-501A amplifier as previously described (Gereau & Conn, 1995*a*, *b*). Electrodes were pulled from borosilicate glass on a Narashige PP-83 vertical puller to a resistance of 3–6 MΩ, and were filled with solution containing (mM): 40 Hepes, 100 gluconic acid, 0·6 EGTA, 0·3 GTP, 2 ATP, 5 $MgCl_2$, pH adjusted to 7·4 with 50% CsOH. Series resistance was monitored during the course of experiments, and cells with a series resistance of >25 MΩ, or that changed by greater than 20%, were discarded. For recording excitatory synaptic currents, 100 μM picrotoxin was included in the ACSF to block inhibitory synaptic currents. In addition, an incision was made between areas CA1 and CA3 to reduce recurrent excitation.

Intracellular and extracellular recordings were obtained with an Axoclamp 2A amplifier (Axon Instruments). Sharp microelectrodes were pulled on a Flaming–Brown electrode puller (Sutter Instruments, San Rafael, CA, USA) from 1·2 mm borosilicate glass (World Precision Instruments, Sarasota, FL, USA), filled with 3 M KCl (90–140 MΩ) or 2 M potassium methylsulphate (135–180 MΩ) and advanced into the CA1 pyramidal cell layer in 2·5–5·0 μm steps. Cells were penetrated by brief overutilization of capacitance compensation and were accepted for use if resting membrane potential was more negative than −60 mV and action potentials were overshooting. Bridge balance was monitored periodically throughout experiments. Slow after-hyperpolarizations (AHPs) were elicited by brief (80–130 ms) depolarizing current injection (0·3–0·7 nA). These depolarizations were routinely monitored to ensure a consistent number of action potentials throughout the experiment. To maintain a constant resting membrane potential throughout the experiment, DC current was injected as necessary. AHP amplitude was monitored until stable for at least 10 min before drug was applied. For extracellular recordings, 2 M NaCl-filled electrodes (1–3 MΩ) were positioned in the stratum radiatum of area CA1. A bipolar tungsten stimulating electrode was also placed in the stratum radiatum, for stimulation of SC afferents (0·1 ms). Drugs were applied through the perfusion medium. Data were digitized and stored on hard disk and analysed using pCLAMP data acquisition and analysis software (Axon Instruments).

Histology

For Nissl-staining of kainate-treated and control hippocampal slices (400 μm thickness), tissue was placed in 37% formalin after incubation with kainate or ACSF. After 24 h, the slices were transferred to a 30% sucrose solution for another 24 h, following which the slices were subsectioned at 30 μm thickness on a cryostat. Subsectioned slices were then mounted on Superfrost slides (Fisher, Pittsburgh, PA, USA), allowed to dry, dehydrated, stained with 0·1% Cresyl Violet, and destained appropriately with acid alcohol. Slides were then coverslipped and viewed on a Nikon Microphot-FXA microscope.

Materials

DCG-IV was generously provided by Dr Haruhiko Shinozaki (Tokyo Metropolitan Institute for Medical Science, Tokyo). 1*S*,3*S*-ACPD, (+)-α-methyl-4-carboxyphenylglycine ((+)-MCPG), 2*S*,3*S*,4*S*-2-methyl-2-(carboxycyclopropyl) glycine (MCCG) and 2*S*,3*S*,4*S*-α-(carboxycyclopropyl) glycine ((−)-CCG-1) were purchased from Tocris Neuramin (Bristol, UK). All other drugs were purchased from Sigma. Fluorocitrate was purchased as the barium salt, which was then replaced with sodium as previously described (Paulsen, Contestabile, Villani & Fonnum, 1987).

RESULTS

Potentiation of Iso-stimulated cAMP accumulation and subsequent depression of synaptic transmission at the SC–CA1 synapse by DCG-IV are both blocked by antagonists of group II mGluRs

Consistent with previous reports, application of DCG-IV (1 μM) had no effect on excitatory postsynaptic currents (EPSCs) recorded from CA1 pyramidal cells in response to Schaffer collateral stimulation (Gereau & Conn, 1995*b*, Gereau *et al.* 1995). Furthermore, application of Iso (1 μM) alone resulted in an increase in EPSC amplitude, as previously reported (Gereau & Conn, 1994*b*,*c*). However, coapplication of Iso plus DCG-IV resulted in a robust depression of EPSCs (Fig. 1; Gereau & Conn, 1994*b*; Gereau *et al.* 1995). Although the agonist profile for activation of the mGluR coupled to potentiation of cAMP accumulation and depression of synaptic transmission when coactivated with βARs strongly suggests the involvement of group II mGluRs (Winder & Conn, 1993; Winder *et al.* 1993; Gereau *et al.* 1995; Winder & Conn, 1995), until recently, no subtype-selective mGluR antagonists were available. Several group II mGluR antagonists have now been described. For instance, (+)-MCPG is an antagonist at both mGluR-1 and mGluR-2, but not mGluR-4 in transfected cell lines (for review see Watkins & Collingridge, 1994). MCCG is a recently described mGluR antagonist that appears to be selective for group II mGluRs (Jane, Jones, Pook, Tse & Watkins, 1994; Knopfel, Lukic, Leonardt, Flor, Kuhn & Gasparini, 1995). In order to test further the hypothesis that the potentiation of cAMP accumulation and resultant depression of excitatory synaptic transmission are mediated by a group II mGluR, we tested the ability of these compounds to inhibit the large increase in cAMP accumulation and depression of synaptic transmission elicited by coapplication of Iso and DCG-IV. The potentiation of Iso-stimulated cAMP accumulation by DCG-IV was antagonized in a concentration-dependent fashion by both (+)-MCPG and MCCG, with approximate IC_{50} values of 300 and 50 μM, respectively (Fig. 2*C*). Likewise, the depression of transmission by coapplication of DCG-IV and Iso was antagonized by (+)-MCPG (1 mM) and MCCG (250 μM) (Fig. 2*A* and *B*). The finding that antagonists of group II mGluRs inhibit potentiation of cAMP accumulation and the depression of synaptic transmission induced by DCG-IV plus Iso is consistent with a body of previously reported agonist pharmacology that suggests that these effects are mediated by a group II mGluR (Gereau & Conn, 1994*b*; Gereau *et al.* 1995; Winder & Conn, 1995).

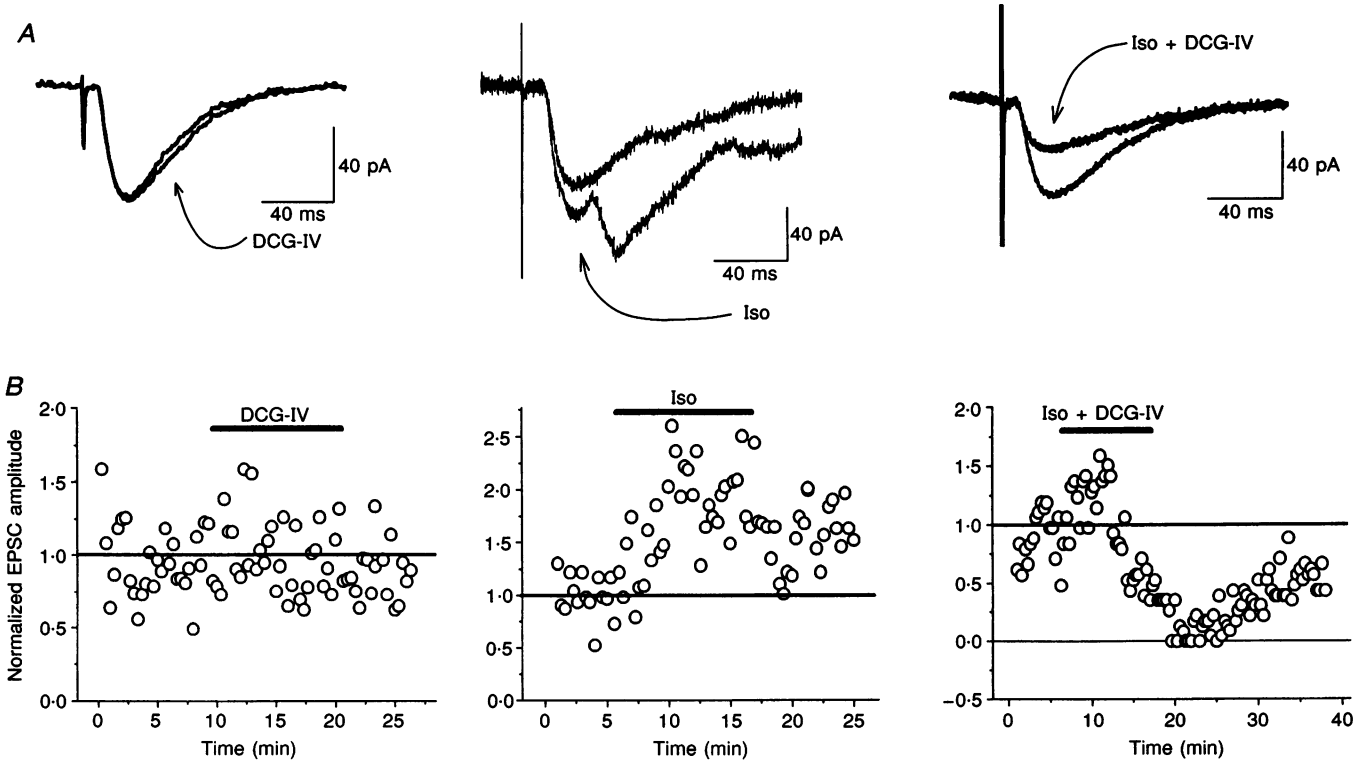

Figure 1. Effects of application of DCG-IV and Iso, alone and in combination, on excitatory synaptic responses at the SC–CA1 synapse

Representative experiments in which DCG-IV (1 μM), Iso (1 μM), or DCG-IV plus Iso were applied to rat hippocampal slices. Pre-drug EPSC amplitudes were always below 100 pA to reduce series resistance error. These results are in agreement with previously published reports (Gereau & Conn, 1994*a*,*b*, 1995*b*; Gereau *et al.* 1995).

Application of DCG-IV fails to enhance cAMP-mediated phenomena in CA1 pyramidal cells

If mGluRs coupled to potentiation of cAMP accumulation are present on CA1 pyramidal cells, the principal neurons in area CA1, it is likely that activation of these receptors would enhance cAMP-mediated effects modulated by G_s-coupled receptors in these cells. One such effect of cAMP in CA1 pyramidal cells is inhibition of the slow after-hyperpolarization (AHP) that follows a burst of action potentials. The slow AHP is mediated by a calcium-dependent potassium conductance, and is inhibited by manipulations that increase cAMP levels, including activation of βARs by Iso (Madison & Nicoll, 1986).

As shown in Fig. 3, application of Iso (30 nM) resulted in a slight inhibition of the slow AHP (31 ± 2% reduction, $n = 16$). A second application of Iso in the same cell also resulted in inhibition of the slow AHP but, to a lesser extent, suggesting a slight desensitization of the response with repeated drug applications (Fig. 3*A* and *B*; 18 ± 7% reduction, $n = 5$). While 30 nM Iso induced only a slight inhibition of the slow AHP, higher concentrations of Iso can almost completely suppress this potential (Fig. 6; Madison & Nicoll, 1986).

Interestingly, the selective group II mGluR agonist DCG-IV failed to potentiate Iso induced inhibition of the AHP. Thus, the response to Iso in the presence of DCG-IV (1 μM; a maximally effective concentration at potentiation of cAMP accumulation; Winder & Conn, 1995) was indistinguishable from repeated application of Iso alone (Fig. 3*E* and *F*; 33 ± 6% reduction for Iso alone; 15 ± 2% reduction for Iso

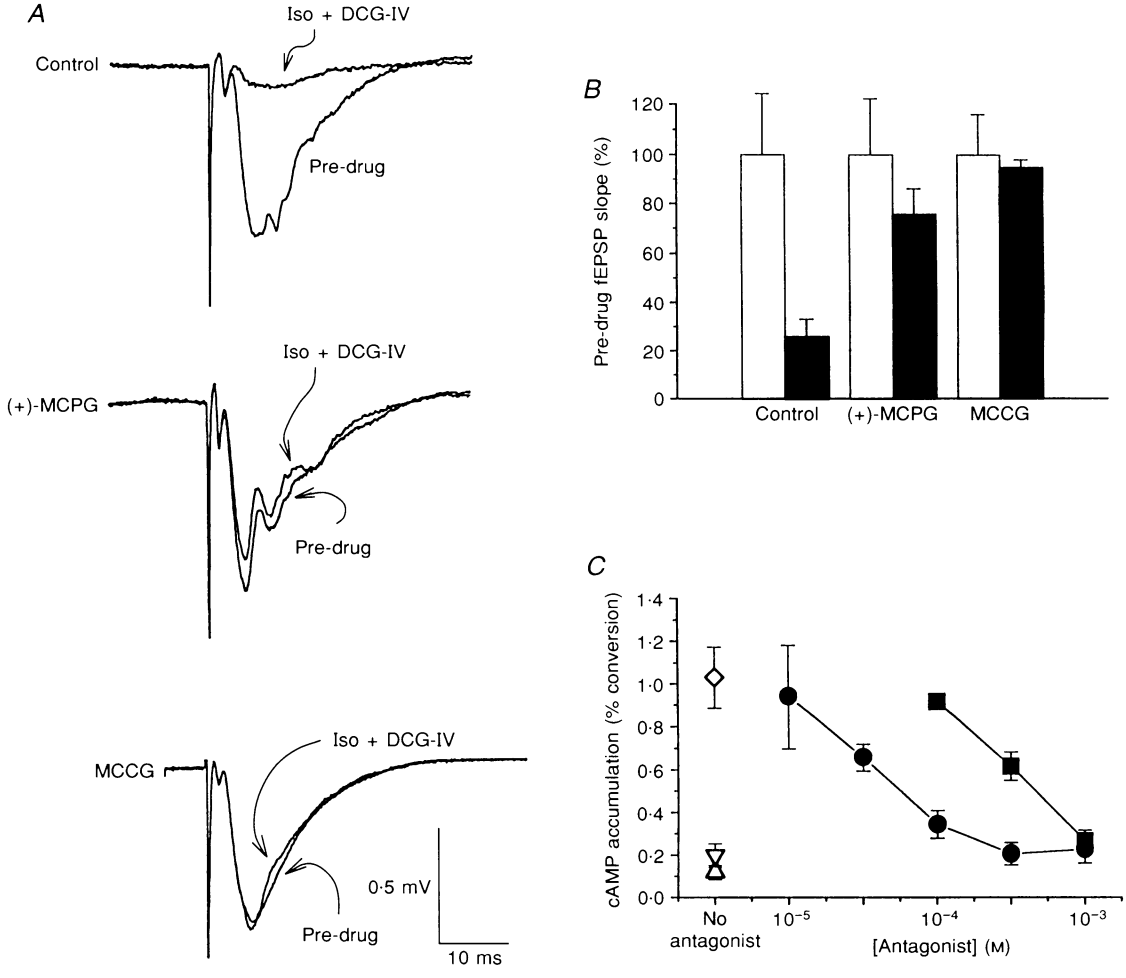

Figure 2. Effects of mGluR antagonists on DCG-IV plus Iso-stimulated cAMP responses and depression of synaptic transmission at the SC–CA1 synapse

A, representative fEPSPs recorded at the SC–CA1 synapse before (Control) and 10 min after application of 1 μM Iso plus 1 μM DCG-IV in the presence or absence of 1 mM (+)-MCPG or 250 μM MCCG. Each trace represents the average of three consecutive trials. *B*, mean data for experiments in *A*. □, pre-drug; ■, Iso + DCG-IV; number of experiments, $n = 3$ in each condition. *C*, effects of increasing concentrations of (+)-MCPG (■) and MCCG (●) on the cAMP response elicited by 10 μM Iso alone (▽), and 10 μM Iso plus 200 nM DCG-IV (◇) (no drug, △). Cyclic AMP is measured as the percentage conversion of [^{3}H]adenine to [^{3}H]cAMP. Each symbol represents the mean ± S.E.M. of three experiments, each done in triplicate.

plus DCG-IV). In contrast, the $GABA_B$ receptor agonist baclofen (30 μM) enhanced the ability of 30 nM Iso to inhibit the AHP (Fig. 3*C* and *D*; 28 ± 5% reduction for Iso alone; 41 ± 5% reduction for Iso + Baclofen; $n = 6$; $P < 0{\cdot}05$, Student's paired t test). This is consistent with a previous report by Andrade (1993).

Like group II mGluRs, $GABA_B$ receptors couple to potentiation of cAMP accumulation (Karbon, Duman & Enna, 1984). In addition, these receptors are clearly present on CA1 pyramidal cells (Newberry & Nicoll, 1984). Thus, these data are consistent with the hypothesis that activation of receptors that potentiate Iso-induced increases in cAMP

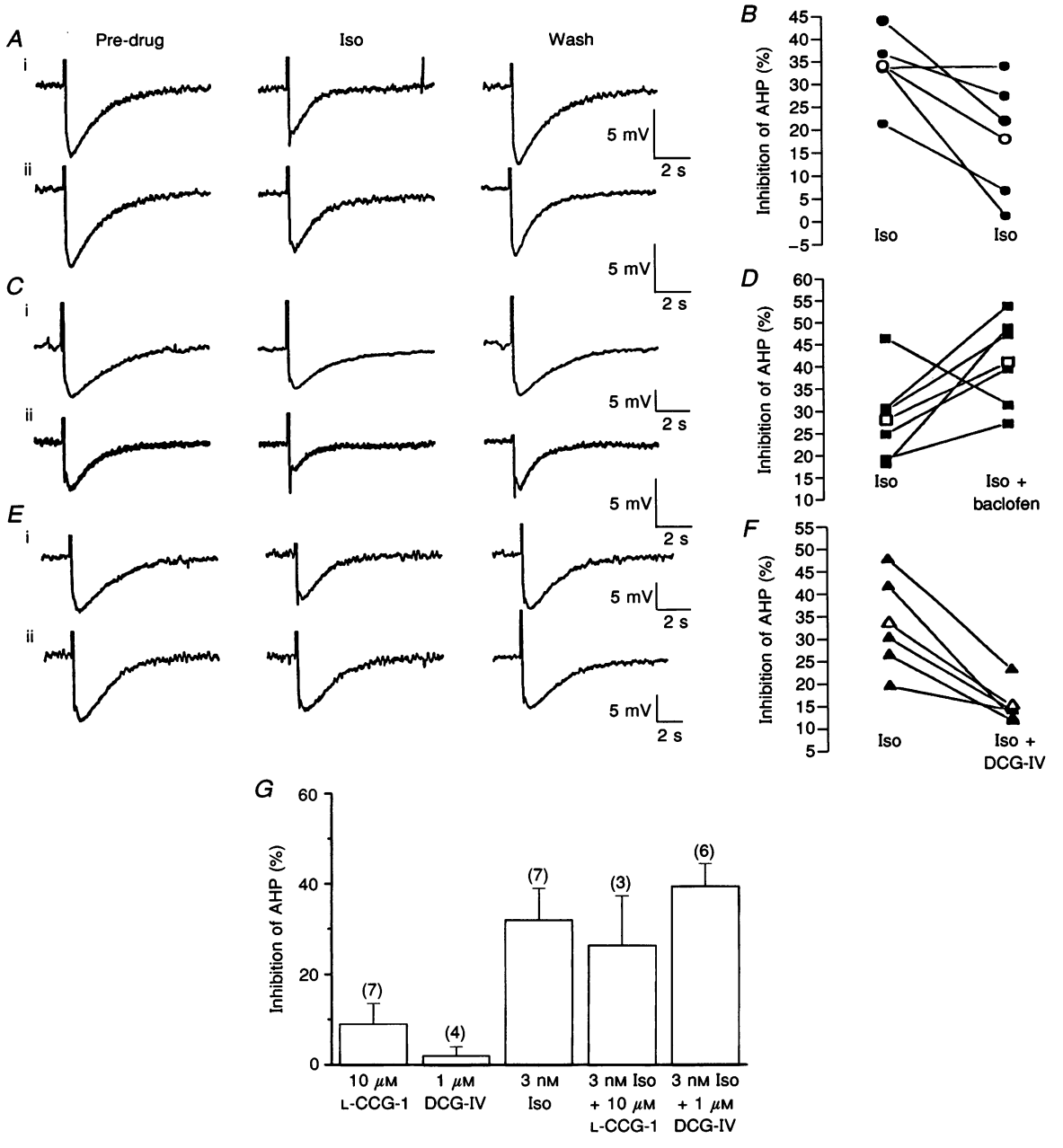

Figure 3. Effects of DCG-IV and baclofen on the inhibition of the slow AHP by Iso

A, *C* and *E*, representative traces. Each trace represents an average of three consecutive trials after either 10 min of drug application, or 10 min of wash. *B*, *D* and *F*, plots of the percentage inhibitions of the AHP in each experiment. The open symbols in each case represent the average response. *A* and *B*, application of 30 nM Iso twice to a CA1 pyramidal cell. *C* and *D*, application of Iso in the absence (i) and the presence (ii) of 30 μM baclofen. *E* and *F*, application of Iso in the absence (i) and presence (ii) of 1 μM DCG-IV. *G*, effects of Iso (3 nM) and Iso (3 nM) plus mGluR agonists on the AHP. Each bar represents the mean ± S.E.M. percentage inhibition of the AHP. The value in parentheses above each bar represents the number of cells tested. Cells in *G* were impaled with KCl electrodes rather than $KMeSO_4$ electrodes.

accumulation in CA1 pyramidal cells can enhance Iso-induced inhibition of the AHP.

Repeated application of Iso to CA1 pyramidal cells results in desensitization of the β-adrenergic receptor-mediated inhibition of the AHP. This desensitization could mask a slight DCG-IV-induced enhancement of the response to Iso. Thus, we confirmed these experiments by comparing the percent inhibition of the AHP by Iso with that elicited by Iso plus DCG-IV in separate populations of cells. Application of Iso, or Iso plus DCG-IV, inhibited the AHP in a statistically indistinguishable fashion (Fig. 3*G*). In addition, L-CCG-1, another group II mGluR agonist that also potentiates cAMP responses (Winder & Conn, 1995), failed to enhance the inhibition of the AHP by Iso (Fig. 3*G*, $n = 3$). Taken together these data suggest that mGluR agonists may not potentiate Iso-induced increases in cAMP accumulation in CA1 pyramidal cells.

Selective glial toxins block mGluR-mediated potentiation of cAMP accumulation

To test the hypothesis that the cAMP increase elicited by coactivation of mGluRs and βARs in area CA1 is localized to glia, hippocampal slices were pre-incubated with the selective glial toxins L-α-amino-adipic acid (L-AA, 1 mM) or fluorocitrate (FC, 100 μM) for various times, after which Iso and 1*S*,3*S*-ACPD, or Iso and DCG-IV were applied and cAMP accumulation measured. FC and L-AA are well-characterized glial toxins that have been widely reported to selectively block glial, but not neuronal, function in cell culture, brain slices and *in vivo* preparations as evidenced by histology, electrophysiology and biochemical markers of glial and neuronal function (Huck, Grass & Hortnagl, 1984; Paulsen *et al.* 1987; Stone, Sessler & Weimin, 1990; Berg-Johnsen, Paulsen, Fonnum & Langmoen, 1993; Keyser & Pellmar, 1994). As shown in Fig. 4, pre-incubation of slices with either toxin resulted in a near complete block of Iso plus 1*S*,3*S*-ACPD-stimulated and Iso plus DCG-IV-stimulated cAMP accumulation within 35 min of toxin pre-incubation, consistent with the hypothesis that mGluRs that potentiate cAMP responses are localized on glia.

To confirm the selectivity of these toxins on glia, sharp microelectrode recordings were made from CA1 pyramidal cells in the presence of L-AA (1 mM) and FC (100 μM) for time periods ranging from 10 to 75 min after toxin application. In all cells tested, neither FC ($n = 5$) nor L-AA ($n = 4$) had any obvious effect on pyramidal cell membrane potential or input resistance. Resting membrane potential was -74 ± 1 mV in control neurons, -71 ± 4 mV in FC treated neurons, and -70 ± 2 mV in L-AA treated neurons. Input resistance was 109 ± 9 MΩ in control neurons, 143 ± 23 MΩ in FC-treated neurons, and 132 ± 11 MΩ in L-AA-treated neurons. Furthermore, we were able to record EPSPs and EPSCs reliably with either extracellular or whole-cell patch electrodes (data not shown). These data are consistent with previous reports that these glial toxins have little or no discernible effects on the viability of neurons (Stone *et al.* 1990; Berg-Johnsen *et al.* 1993).

In addition to these basic aspects of neuronal function, neither FC nor L-AA affected the ability of Iso or forskolin to inhibit the AHP (Fig. 4*A* and *B*). Furthermore, in slices pre-incubated with FC, baclofen was still capable of enhancing Iso-induced inhibition of the AHP ($24 \pm 4\%$ inhibition for Iso alone; $39 \pm 10\%$ inhibition for Iso + Baclofen; $n = 4$; $P < 0{\cdot}05$, Student's paired t test; Fig. 5*C*). Thus, while these toxins inhibit mGluR-mediated potentiation of cAMP accumulation, they have no apparent effect on Iso- or forskolin-stimulated cAMP accumulation, or on $GABA_B$-receptor-mediated potentiation of cAMP accumulation in CA1 pyramidal cells.

Long-term kainate incubation fails to inhibit 1*S*,3*S*-ACPD-stimulated potentiation of cAMP accumulation

Kainic acid is commonly used as a neuronal toxin, and has previously been reported to cause a virtually complete loss

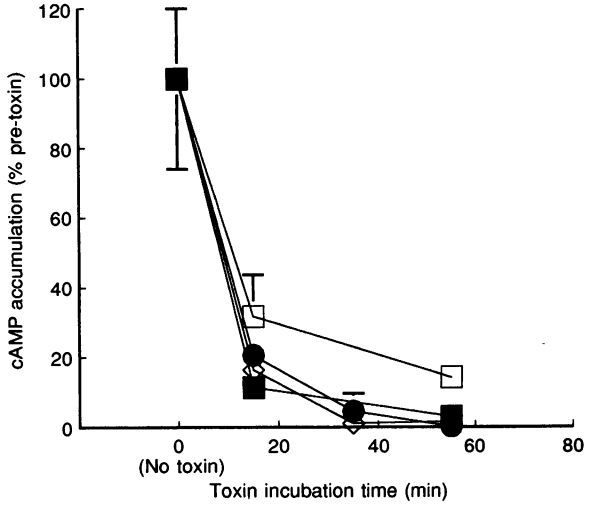

Figure 4. Effects of glial toxins L-α-amino-adipic acid (L-AA, 1 mM) and fluorocitrate (FC, 100 μM) on the cAMP response elicited by 10 μM Iso plus 100 μM 1*S*,3*S*-ACPD or 1 μM DCG-IV

Cross-chopped hippocampal slices were pre-incubated with either glial toxin for 15, 35 and 55 min, the last 15 min of which agonists were applied in the continuing presence of L-AA or FC. ●, Iso + 1*S*,3*S*-ACPD + L-AA; ■, Iso + DCG-IV + L-AA; ◇, Iso + 1*S*,3*S*-ACPD + FC; □, Iso + DCG-IV + FC. Each point represents the mean ± S.E.M. of three separate experiments, each done in triplicate.

of neurons from the cerebellar slice preparation with a 2 h incubation (Garthwaite & Wilkin, 1982). We took advantage of this toxin to determine whether disruption of neuronal function would inhibit mGluR-mediated potentiation of Iso-induced cAMP responses in hippocampal slices. Thus, we incubated transverse hippocampal slices with 100 μM kainate for 3 h to elicit excitotoxic death of neurons. Pretreatment of hippocampal slices with kainate in this manner resulted in marked degeneration of neurons in area CA1 (Fig. 6*C*). In contrast to control hippocampal slices treated in parallel, few, if any recognizable neurons were present in the hippocampus proper in slices treated with kainate. Instead, small dark spheres remained, which are probaby pyknotic nuclei based on Haematoxylin and Eosin staining (data not shown). There was a virtually complete loss of both pyramidal cells and non-pyramidal cells in all major subsectors of hippocampus proper (CA1–CA3). Marked neurodegeneration was also seen in dentate gyrus, though there was evidence of some sparing of neurons within this region (data not shown). Consistent with neuronal degeneration in area CA1 and CA3, pre-incubation with kainate abolished excitatory synaptic transmission of the SC–CA1 pyramidal cell synapse (Fig. 6*B*). While there was clear loss of neurons in these slices, the ability of 1*S*,3*S*-ACPD and Iso to increase cAMP responses was not decreased, consistent with the hypothesis that the bulk of this response is mediated by glial receptors (Fig. 6*A*).

Blockade of cAMP/cAMP metabolite efflux results in a blockade of the depression of synaptic transmission elicited by coactivation of group II mGluRs and βARs

The data presented here suggest that glially localized increases in cAMP accumulation induced by coactivation of group II mGluRs and β-adrenergic receptors results in the depression of excitatory synaptic transmission at the SC–CA1 synapse. Consistent with these data, Rosenberg, Knowles & Li (1994) found that cultured cortical glia, but not neurons, release cAMP and cAMP metabolites in response to β-adrenergic receptor activation. This release of cAMP and cAMP metabolites was reduced by the anion transport blocker probenecid (Rosenberg *et al.* 1994). Therefore, we tested the ability of probenecid (1 mM) to block the depression of fast EPSPs (fEPSPs) elicited by coapplication of DCG-IV (1 μM) and Iso (1 μM). As shown in Fig. 7, in hippocampal slices pretreated with probenecid, coapplication of DCG-IV and Iso failed to reduce fEPSPs

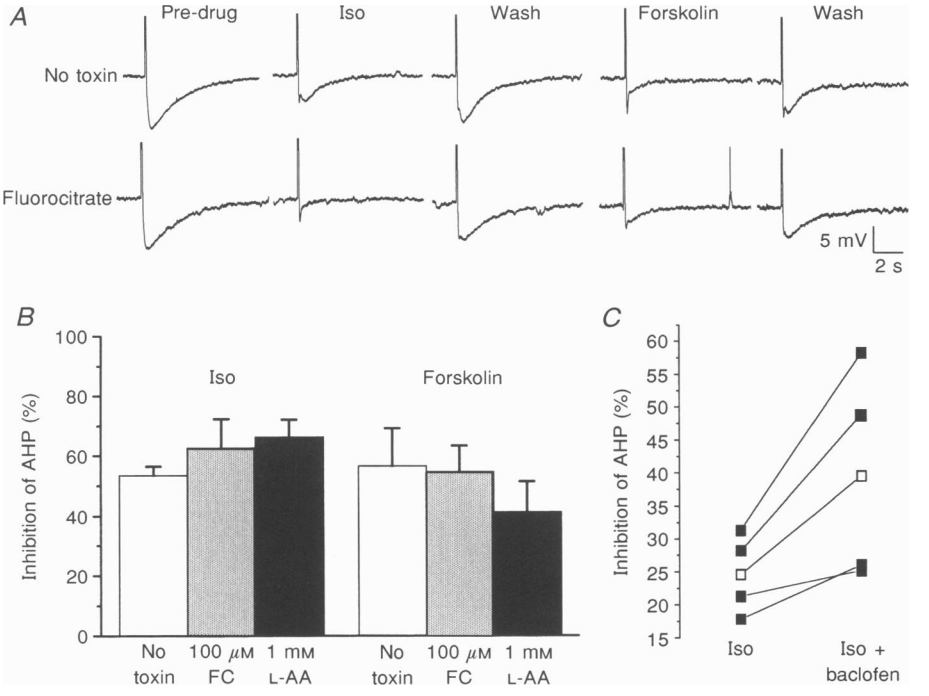

Figure 5. Effects of L-AA (1 mM) and FC (100 μM) on modulation of the AHP in CA1 pyramidal cells

A, traces from representative cells recorded in the presence or absence of FC. Each trace represents the average of three consecutively elicited AHPs. After recording a stable baseline, 100 nM Iso was applied for 10 min and washed out, followed by application of 10 μM forskolin. *B*, mean data for all cells tested. Each bar represents the S.E.M. percentage inhibition of the AHP in three to five separate cells. *C*, effects of 30 μM baclofen on the inhibition of the AHP by 30 nM Iso in slices pre-incubated with 100 μM FC. The open symbols show the average response in each condition.

($n = 3$). This concentration of probenecid had no effect on the ability of adenosine to block excitatory synaptic transmission, suggesting that probenecid was not acting through direct modulation of adenosine receptors. Furthermore, this concentration of probenecid had no significant effect on the increased cAMP accumulation elicited by Iso and DCG-IV, suggesting that the effect on synaptic transmission was through blockade of release of cAMP or a cAMP metabolite rather than decreasing cAMP production (Fig. 7)

DISCUSSION

Glial localization of mGluR-mediated potentiation of cAMP accumulation suggests a novel glial–neuronal interaction

One of the primary functions of mGluRs in the CNS is to modulate neuronal excitability and synaptic transmission (for review see Pin & Duvoisin, 1995). In most cases, this modulation is thought to be accomplished by activation of presynaptic mGluRs which affect neurotransmitter release, and/or postsynaptic mGluRs that regulate membrane

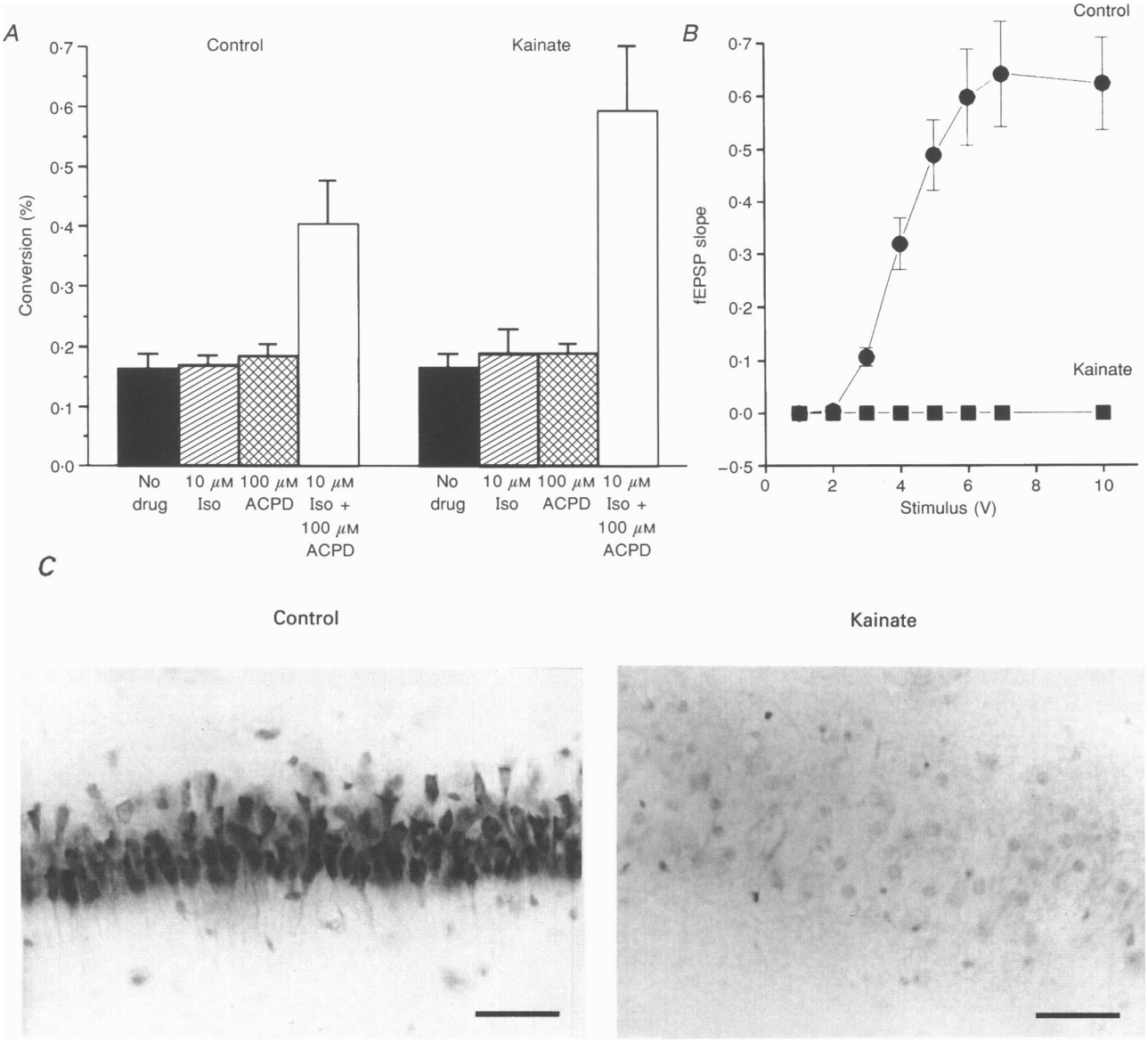

Figure 6. Effects of a 3 h kainate (100 μM) incubation on mGluR-mediated cAMP responses and synaptic transmission at the SC–CA1 synapse

A, mean ± S.E.M. cAMP responses elicited by various drugs in slices pre-incubated with either kainate or ACSF. ACPD, 1*S*,3*S*-ACPD. *B*, input–output curve for fEPSPs recorded at the SC–CA1 synapse of hippocampal slices pre-incubated in kainate or ACSF. Each point represents the mean ± S.E.M. of fEPSP slopes determined from three slices incubated on different days. *C*, Nissl-stained 30 μm thick sections from control and kainate-treated slices showing area CA1. These images are representative of those seen in twenty control slice subsections and twenty kainate-treated slice subsections from three separate control and kainate-treated slices. Bars indicate 10 μm.

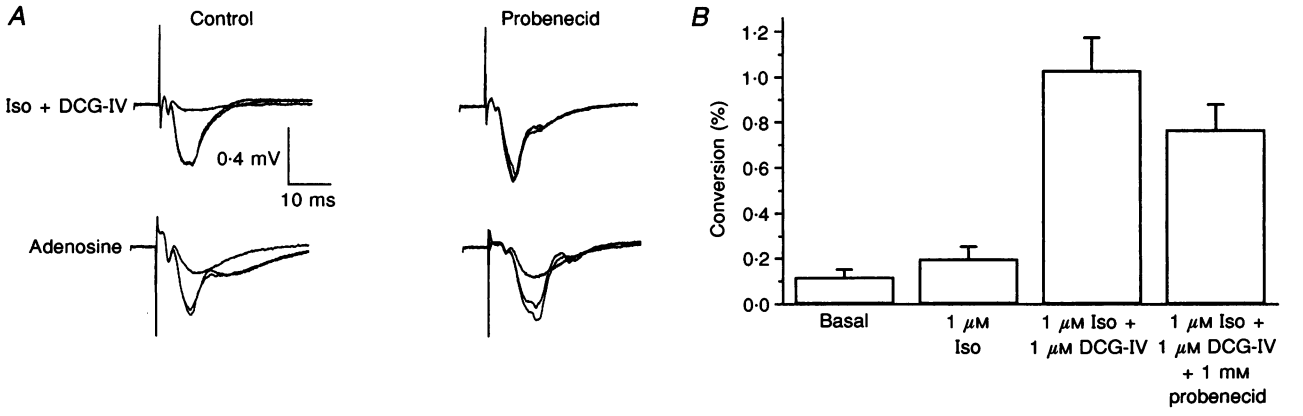

Figure 7. Effects of probenecid on DCG-IV plus Iso-stimulated cAMP responses and depression of synaptic transmission at the SC–CA1 synapse

Hippocampal slices were pretreated with probenecid (1 mM), after which Iso (1 μM) plus DCG-IV (1 μM), or adenosine (100 μM) was applied. *A*, representative experiments showing that probenecid blocked the depression of synaptic transmission elicited by Iso and DCG-IV ($n = 3$). *B*, mean data for the effects of probenecid on the cAMP response elicited by Iso plus DCG-IV. Each bar represents the mean + S.E.M. of three experiments, each done in triplicate.

excitability. However, we previously reported evidence for a novel mechanism by which mGluR activation can regulate excitatory synaptic transmission in the CNS. Activation of mGluRs in conjunction with receptors directly positively coupled to adenylyl cyclase leads to a large increase in cAMP levels, which results in the release of cAMP metabolites into the extracellular space, where they activate presynaptic A_1 adenosine receptors to depress synaptic transmission at the SC–CA1 synapse (Winder & Conn, 1992; Gereau & Conn, 1994*b*). The data presented in the present paper suggest that the receptors that mediate this response are localized on glia and not CA1 pyramidal cells in the hippocampus (Fig. 8).

Several lines of evidence support this hypothesis. First, extensive pharmacological studies suggest that this response is mediated by a group II mGluR (Winder *et al.* 1993; Winder & Conn, 1995; Gereau *et al.* 1995; present data), and *in situ* hybridization studies suggest that group II mGluRs are probably present only in glia in area CA1 of hippocampus, although immunocytochemical analysis will be needed to more definitively state the distribution of these receptors (Testa *et al.* 1994). Second, activation of mGluRs that potentiate cAMP responses in hippocampus did not enhance cAMP-mediated electrophysiological responses in CA1 pyramidal cells, while activation of other receptors coupled to potentiation of cAMP accumulation ($GABA_B$

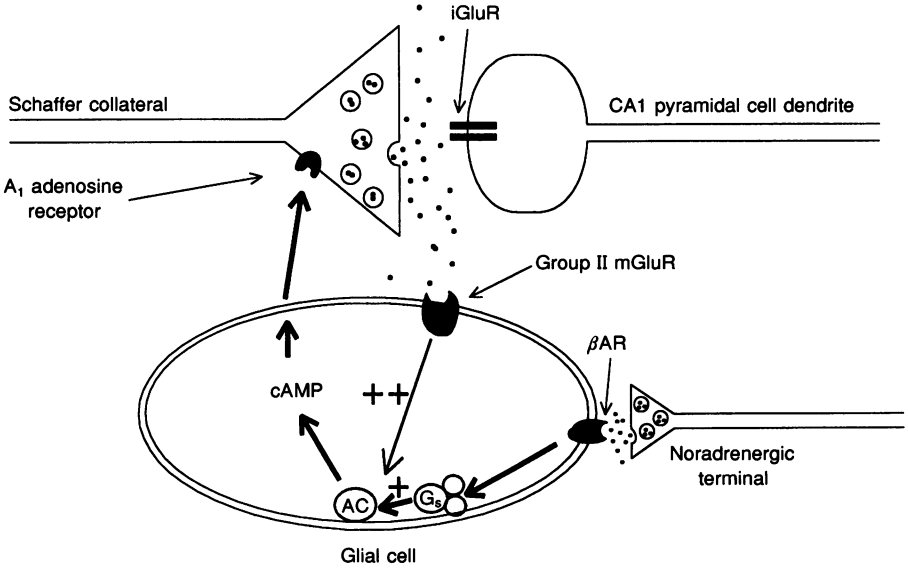

Figure 8

Model of the location of mGluRs coupled to potentiation of cAMP responses and their potential physiological significance. iGluR, ionotropic glutamate receptor.

receptors) did. Third, manipulations that selectively disrupt glial function blocked the cAMP response elicited by coactivation of βARs and mGluRs in hippocampus. Finally, disruption of neuronal function by pre-incubation of hippocampal slices with kainate failed to block the cAMP response mediated by coactivation of βARs and mGluRs. Taken together, these studies provide strong support for a glial localization of mGluR-mediated potentiation of cAMP accumulation. Furthermore, recent data suggest that in striatum, activation of mGluRs does not potentiate the cAMP accumulation elicited by D_1/D_5 dopamine receptors that are commonly thought to be neuronally localized (Wang & Johnson, 1995). However, activation of mGluRs in striatum does potentiate cAMP accumulation elicited by A_2 adenosine receptors, which are present on glia, as well as neurons (Winder, Smith & Conn, 1993; Peakman & Hill, 1994; Wang & Johnson, 1995). Consistent with this finding, mGluR-mediated potentiation of cAMP accumulation in striatum is also decreased by pre-incubation of slices with glial toxins (data not shown). Thus, it will be interesting to determine whether a similar type of modulation of excitatory synaptic transmission to that discussed above in hippocampus is present in striatum as well.

As mentioned, the large increase in cAMP accumulation induced by coactivation of mGluRs and β-adrenergic receptors leads to a release of cAMP, or a cAMP metabolite (i.e. adenosine) into the extracellular space, and activation of A_1 adenosine receptors (Gereau & Conn, 1994*b*). Activation of A_1 adenosine receptors in hippocampus results in inhibition of release of glutamate (Dunwiddie & Hoffer, 1980). Interestingly, it has previously been reported that activation of βARs in primary glial cultures results in the efflux of cAMP and its metabolites into the extracellular medium (Rosenberg *et al.* 1994). This release of cAMP/cAMP metabolites has been hypothesized to play a role in neuronal signalling. Indeed we found that pretreatment of hippocampal slices with a compound that inhibits efflux of cAMP and its metabolites from cultured glia blocks the depression of synaptic transmission elicited with coapplication of Iso and DCG-IV. Unfortunately, it was not possible to assess the effects of the glial toxins on the depression of synaptic transmission by activation of this effector system, because pre-incubation of hippocampal slices with glial toxins resulted in recordable, but highly unstable EPSCs, as has been previously reported (Keyser & Pellmar, 1994). These findings are consistent with previous data showing the critical role of glia in the glutamate–glutamine cycle, as well as in maintenance of potassium homeostasis (for review see Hansson & Ronnback, 1995). Thus, in future studies, it will be important to determine the physiological circumstances under which this effector system is activated. In total, however, the present data in combination with previously reported data provide evidence for the model presented in Fig. 8, in which there is a novel associative form of glial–neuronal interaction that is clearly distinct from other reported mechanisms for glial–neuronal signalling (for review see Smith, 1994).

It is important to note that, in another study, application of 1*S*,3*S*-ACPD alone induced a depression of synaptic transmission at the SC–CA1 synapse in neonatal Wistar rats (14 days old), and it was proposed that this response was mediated by a group II mGluR (Vignes *et al.* 1995). Consistent with this, we found a similar depression of synaptic transmission by application of group II mGluR agonists alone in neonatal rat hippocampal preparations (R. W. Gereau IV & P. J. Conn, unpublished observations), suggesting a developmental regulation of group II autoreceptors in area CA1. However, neither DCG-IV nor low concentrations ($< 100\ \mu M$) of 1*S*,3*S*-ACPD depress excitatory transmission at this synapse in adult rat (Desai, Smith & Conn 1992; Gereau *et al.* 1995; Gereau & Conn, 1995*b*). Thus, the studies reported here are from 31- to 45-day-old Sprague–Dawley rats.

Ultrastructural localization of βARs is consistent with the proposed model

Previous anatomical studies provide interesting insights that are relevant to the model presented in Fig. 8. For instance, astrocytic processes are often seen to envelope synapses in CNS (Peters, Palay & Webster, 1991). Furthermore, ultrastructural analysis reveals that astrocytic processes exhibiting βAR-immunoreactivity envelope both catecholaminergic and non-catecholaminergic synapses in many cortical and non-cortical structures (Aoki, 1992), and the βAR immunoreactivity on astrocytic processes is often in close apposition to catecholaminergic terminals (Aoki, 1992). Interestingly, the non-catecholaminergic synapses that are enveloped by astrocytic processes containing βARs have a morphology that is consistent with excitatory synapses and are thought to be glutamatergic synapses. If the group II mGluRs that potentiate cAMP responses are co-localized with βARs on astrocytic processes, this would place them in an ideal position to regulate cAMP levels in glia in the vicinity of excitatory synapses and reduce excitatory synaptic transmission by the mechanism outlined in Fig. 8. While it is clear from the *in situ* hybridization studies that group II mGluRs are present in glia future immunocytochemical studies and electron microscopic analysis will be needed to determine whether these receptors are co-localized with βAR on astrocytic processes.

Regulation of βAR responses by mGluRs in CNS

The finding that DCG-IV does not potentiate Iso-induced inhibition of the slow AHP in CA1 pyramidal cells does not entirely rule out the possibility that mGluRs that potentiate cAMP responses are present in these cells. However, the finding that activation of $GABA_B$ receptors did enhance the Iso-induced inhibition of the slow AHP (Andrade, 1993; present results) suggests that receptors coupled to potentiation of cAMP accumulation that are present in CA1 pyramidal cells do enhance the effects of Iso on the slow AHP. Furthermore, as mentioned above, previous *in situ*

hybridization studies suggest that group II mGluRs are not present in CA1 pyramidal cells (Testa *et al.* 1994). Furthermore, we have previously reported that DCG-IV does not potentiate a cAMP-dependent long-lasting increase in CA1 pyramidal cell excitability induced by transient βAR activation with high concentrations of Iso (β-adrenergic receptor potentiation, βAP; Heginbotham & Dunwiddie, 1991; Gereau *et al.* 1995).

Interestingly, while DCG-IV failed to potentiate βAP, another mGluR agonist, 1*S*,3*S*-ACPD, enhanced the ability of submaximal concentrations of Iso to elicit βAP (Gereau & Conn, 1994*a*; Gereau *et al.* 1995). Since DCG-IV does not mimic this effect of 1*S*,3*S*-ACPD it is probably not mediated by a group II mGluR. However, this response was mimicked by the group I mGluR-selective agonist DHPG (Gereau *et al.* 1995). At present, the mechanism by which DHPG and 1*S*,3*S*-ACPD enhance βAP is unknown. One possibility is that a group I mGluR exists in CA1 pyramidal cells that also potentiates cAMP responses, perhaps through activation of protein kinase C (PKC).

Glial–neuronal signalling

Glial cells have long been assumed to function solely in the physical and metabolic support of neurons. Recently however, this view has been called into question by several observations. (1) Glia cells express on their surface a variety of neurotransmitter receptors (for review see Smith, 1994). (2) When properly stimulated, calcium waves can be elicited in glia that then spread to neighbouring glia and also to neurons. (3) Stimulation of glia in culture leads to the release of glutamate and subsequent activation of glutamatergic receptors on neurons (for review see Smith, 1994). (4) Activation of βARs on cultured glia regulates extracellular adenosine levels (Rosenberg *et al.* 1994). Based on these findings, it has been proposed that glia may have a more prominent role than was originally assumed in signal processing in the CNS. The present studies add to this growing body of literature and provide a novel associative mechanism for a glial–neuronal interaction that is clearly distinct from other forms of glial–neuronal communication that have been reported to date.

Conclusion

In summary, we have provided converging lines of evidence that suggest that coactivation of mGluRs and βARs on glia in area CA1 of hippocampus induces a large synergistic increase in cAMP accumulation. This provides a novel mechanism by which glia can regulate synaptic transmission through the release of cAMP, or a cAMP metabolite, and activation of adenosine receptors on nearby glutamatergic terminals, and provides further evidence that glia play important roles in modulation of synaptic transmission. Future studies will be needed to test the hypothesis that this response can be mimicked by more physiological stimuli, such as coactivation of noradrenergic and glutamatergic afferents in the hippocampus.

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