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Protein Kinase C and A_3 Adenosine Receptor Activation Inhibit Presynaptic Metabotropic Glutamate Receptor (mGluR) Function and Uncouple mGluRs from GTP-Binding Proteins

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One of the most prominent roles of metabotropic glutamate receptors (mGluRs) in the CNS is to serve as presynaptic receptors that inhibit transmission at glutamatergic synapses. Previous reports suggest that the presynaptic effect of group II mGluRs at corticostriatal synapses can be inhibited by activators of protein kinase C (PKC). We now report that activation of PKC inhibits the ability of group II and group III mGluRs to regulate transmission at three major synapses in the hippocampal formation. Thus, this effect may be a widespread phenomenon that occurs at glutamatergic synapses throughout the CNS. We also report that this response is not limited to PKC-activating phorbol esters but that activation of A_3 adenosine receptors induces a PKC-dependent inhibition of group III mGluR function at the Schaffer collateral–CA1 synapse. In addition to inhibiting mGluR modulation of excitatory synaptic transmission, we found that activation of PKC reduces inhibition of forskolin-stimulated cAMP accumulation by group II and group III mGluRs, suggesting that the effect of PKC on mGluR signaling is not specific to their effects on neurotransmitter release. This led us to test the hypothesis that PKC acts upstream from effector proteins regulated by mGluRs and acts at the level of the receptor or GTP-binding protein. Interestingly, we found that PKC inhibited mGluR-induced increases in [^{35}S]-GTPγS binding in cortical synaptosomes. These data suggest that PKC-induced inhibition of mGluR signaling may be mediated by the inhibition of coupling of mGluRs to GTP-binding proteins.

Key words: metabotropic glutamate receptor (mGluR); protein kinase C (PKC); hippocampus; cAMP; A_3 adenosine receptors; GTPγS binding; synaptic transmission

Throughout the CNS both cell excitability and fast synaptic transmission are modulated by the activation of a family of G-protein-coupled receptors termed metabotropic glutamate receptors (mGluRs). Eight mGluR subtypes have been cloned to date and have been classified into three major groups on the basis of sequence homology, effector coupling, and pharmacological properties. Group I mGluRs (mGluR1 and mGluR5) couple primarily to phosphoinositide hydrolysis, whereas group II (mGluR2 and mGluR3) and group III (mGluRs 4, 6, 7, and 8) couple to the inhibition of adenylyl cyclase in expression systems (for review, see Pin and Duvoisin, 1995; Conn and Pin, 1997).

One of the primary functions of mGluRs seen throughout the CNS is to serve as presynaptic receptors involved in reducing transmission at glutamatergic synapses. Activation of presynaptic mGluRs has been shown to reduce transmission at glutamatergic synapses in a wide variety of brain regions, including the hippocampus, amygdala, olfactory cortex, neocortex, spinal cord, striatum, cerebellum, nucleus of the solitary tract, and olfactory bulb. mGluR activation reduces transmission at glutamatergic synapses in each major subsector of the hippocampus, and different complements of mGluR subtypes serve this role at each of the three major excitatory hippocampal synapses (for review, see Glaum and Miller, 1994; Conn and Pin, 1997).

Previous studies (Swartz et al., 1993; Tyler and Lovinger, 1995) revealed that activation of protein kinase C (PKC) can reduce dramatically the presynaptic inhibitory function of group II mGluRs at corticostriatal synapses. This effect of PKC could play a critical role in fine-tuning transmission at glutamatergic synapses. Furthermore, selective agonists or antagonists of presynaptic receptors that activate PKC could provide novel therapeutic targets for the development of drugs that could be used to regulate transmission at glutamatergic synapses. However, it is not yet clear whether PKC-induced inhibition of mGluR function is restricted to the modulation of group II mGluRs or is a more general phenomenon. Also, it is not clear whether this response can be elicited only with PKC activators, such as phorbol esters, or whether it is a physiologically relevant response that can be elicited by agonists of receptors coupled to activation of phosphoinositide hydrolysis (and PKC). Finally, there is little understanding of the molecular mechanisms by which PKC inhibits mGluR function. We now report that activators of PKC disrupt presynaptic inhibitory functions of mGluRs at several synapses in the hippocampal formation, including the lateral perforant path (LPP)–dentate gyrus synapse, the medial perforant path (MPP)–dentate gyrus synapse, and the Schaffer collateral–CA1 (SC–CA1) synapse. This includes responses mediated by a group II mGluR and at least two group III mGluR subtypes. Furthermore, we find that activation of A_3 adenosine receptors reduces mGluR-mediated inhibition at the SC–CA1 synapse by a PKC-dependent mechanism. Finally, we report that PKC inhibits the coupling of

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group II and group III mGluRs to multiple signaling pathways by disrupting the coupling of mGluRs to GTP-binding proteins.

MATERIALS AND METHODS

Materials. (2*S*,2′*R*,3′*R*)-2-(2′ 3′-dicarboxycyclopropyl)glycine (DCG-IV) (Ishida et al., 1993) was generously supplied by Drs. H. Shinozaki (Tokyo Metropolitan Institute for Medical Sciences, Tokyo, Japan) and Y. Ohfune (Suntory Institute of Bioorganic Research, Osaka, Japan) or purchased from Tocris Cookson (Bristol, UK). 2-Chloro-N^6-(3-iodobenzyl)-adenosine-5-*N*-methyluronamide (Cl-IB-MECA) was provided by Research Biochemicals (Natick, MA) as part of the Chemical Synthesis Program of the National Institute of Mental Health, contract N01MH30003. 3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS 1191) was a generous gift from Dr. Kenneth Jacobson (National Institutes of Health, Bethesda, MD). Bisindolylmaleimide I-HCl (Bis) was purchased from Calbiochem (San Diego, CA). (L)-2-Amino-4-phosphonobutyric acid (L-AP4) was purchased from Tocris Cookson. [^{35}S]-GTPγS was purchased from NEN-DuPont (Boston, MA), [^{3}H]-adenine was purchased from American Radiolabeled Chemicals (St. Louis, MO), and adenosine deaminase was obtained from Boehringer Mannheim (Indianapolis, IN). All other chemicals were purchased from Sigma (St. Louis, MO). MRS1191, Bis, phorbol-12,13-dibutyrate (PDBu), 4α-phorbol, Cl-IB-MECA, 3-isobutyl-methyl-xanthine (IBMX), and forskolin were all dissolved in DMSO at >100× final concentration and diluted into the final solutions. DMSO concentrations ranged from 0.1 to 1%. All other drugs were dissolved in artificial cerebrospinal fluid (ACSF) containing (in mM) NaCl 124, KCl 2.5, $CaCl_2$ 2, Mg_2SO_4 1.3, NaH_2PO_4 1, $NaHCO_3$ 26, and glucose 10 equilibrated to pH 7.4 with 95% O_2/5% CO_2.

Field potential recordings. Hippocampi from 100–150 gm male Sprague Dawley rats were dissected rapidly on ice, and 400 μM transverse slices were prepared by using a McIlwain tissue chopper. Slices were placed in a holding chamber containing ACSF at room temperature. After a recovery period of at least 1 hr, a slice was transferred to a submerged brain slice recording chamber, where it was perfused continuously with oxygenated ACSF at 1 ml/min and maintained at 33°C. Drugs were delivered via a syringe pump at 10× the final concentration at a rate of 0.1 ml/min. Electrodes were pulled on a Flaming/Brown electrode puller (Sutter Instruments, San Rafael, CA) from 1.2 mm borosilicate glass (World Precision Instruments, Sarasota FL) and filled with 2 mM NaCl. Electrode resistance ranged from 0.2 to 1.0 MΩ. Recording electrodes were stepped slowly through the hippocampal slice with a motorized drive (Newport Corporation, Hamden, CT) until optimal responses were obtained. Stimulus intensity was adjusted to 50% or less of maximal response. Field potential data were acquired by an Axoclamp 2A (Axon Instruments, Foster City, CA) amplifier in bridge mode and stored on a Pentium IBM clone, using pClamp acquisition and analysis hardware and software. Data were filtered at 1 kHz and digitized at >5 kHz. For some experiments a Humbug 50/60 Hz noise eliminator was used to reduce 60 cycle noise (Quest Scientific, North Vancouver, BC, Canada). Effects of agonists and antagonists were determined by averaging the field EPSP (fEPSP) slope from five traces immediately before drug application and comparing them with the fEPSP slope from five traces obtained at the end of drug application. fEPSP slope was determined with software developed by Dr. Stephen Traynelis (Emory University, Atlanta, GA).

For experiments in which fEPSPs were measured at perforant path synapses, stimulating and recording electrodes were placed in either the middle or outer third of the molecular layer of the dentate gyrus for stimulation of either the medial or lateral perforant path. Selective medial or lateral perforant path recordings were made with criteria established previously (see Kahle and Cotman, 1993a,b; Macek et al., 1996). Downward-deflecting field potentials were evoked by a Grass S44 stimulator (0.1 msec; square wave) at ~30 sec intervals. For SC–CA1 recordings, stimulating and recording electrodes were similarly placed in stratum radiatum of area CA1 as described in Gereau and Conn (1995), and stimuli were applied as described above.

Inhibition of cAMP assay. Inhibition of cAMP accumulation was measured by determining the percentage of the conversion of [^{3}H]-adenine to [^{3}H]-cAMP (Shimizu et al., 1969). Cross-chopped hippocampal slices (350 × 350 μm) were prepared from adult male Sprague Dawley rats (125–150 gm) and incubated in Krebs' buffer containing (in mM) NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 10, and $NaHCO_3$ 25 at 37°C for 15 min. Then the tissue was washed and incubated for 40 min in 15 ml of Krebs' buffer containing 30 μCi of [^{3}H]-adenine and 6 μM unlabeled adenine. After several rinses with warmed Krebs' buffer, 25 μl aliquots of gravity-packed slices were transferred to 15 ml reaction tubes. IBMX (200 μM) was included in all reactions, and cAMP was stimulated by the addition of forskolin (30 μM) in the presence of either phorbol esters (PDBu or 4α-phorbol) or vehicle (DMSO) and the appropriate mGluR agonists. The final reaction volume was 0.5 ml. Reactions were terminated after 15 min by the addition of 50 μl 77% trichloroacetic acid, and 25 μl of 10 mM cAMP was added as a carrier. The tissue was homogenized and centrifuged (for 15 min at 17,000 × *g*), and 25 μl of the supernatant was removed for determination of total radioactivity incorporated into the tissue. [^{3}H]-cAMP in the remaining supernatant was isolated by sequential elution through Dowex and then Alumina columns. The inhibition of cAMP accumulation by mGluR agonists was expressed in relation to the percentage of stimulation by forskolin over basal in the absence of mGluR agonists. All reactions were performed in triplicate at 37°C under an atmosphere of 95% O_2/5% CO_2 in a shaking water bath.

Preparation of cortical synaptosomal membranes and measurement of [^{35}S]-GTPγS binding. Cerebral cortices of adult male Sprague Dawley rats were dissected rapidly on ice and cross-chopped (350 × 350 μm) as described above. Cortical tissue was transferred to oxygenated Krebs' buffer and allowed to recover for 1 hr. Equal volumes of gravity-packed slices then were removed and incubated with either vehicle or phorbol esters (PDBu, 10 μM, or 4α-phorbol, 10 μM) for 30 min in separate holding chambers. The slices subsequently were diluted 1:10 (w/v) in ice-cold membrane buffer 1 containing 320 mM sucrose, 20 mM HEPES, and 10 mM EDTA, pH 7.4, and homogenized with a Teflon pestle. The resulting homogenate was centrifuged at 900 × *g* for 10 min. The pellet was discarded and the supernatant was recentrifuged at 48,000 × *g* for 10 min. Then this pellet was resuspended with a Polytron and washed two times with cold membrane buffer 1 and two times with cold membrane buffer 2 containing 20 mM HEPES and 0.1 mM EDTA, pH 7.4. Membranes were stored in aliquots at −80°C. Protein concentration was determined according to the method of Bradford (Pierce BCA reagent, Rockford, IL), with bovine serum albumin as a standard.

On the day of the experiment, aliquots of cortical synaptic membranes were thawed and washed two times in cold assay buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EGTA, and 3 mM $MgCl_2$. After the second centrifugation the pellet was resuspended in assay buffer and homogenized by Polytron to ensure uniform protein concentration. In duplicate, 30–50 μg of protein was added to tubes containing 1 U/ml of adenine deaminase (ADA), 300 μM GDP, and agonists in a volume of 900 μl and incubated at 30°C for 30 min. [^{35}S]-GTPγS (final concentration 0.1 nM) then was added to each tube, and the reaction was incubated for an additional 30 min. Nonspecific binding was defined by using unlabeled GTPγS (10 μM). The reaction was terminated by dilution with ice-cold wash buffer (Tris-HCl 50 mM, pH 7.4) and immediately poured over prewetted Whatman GF/B filters. Bound and free [^{35}S]-GTPγS was separated by vacuum filtration. Then the filters were washed three times with 3 ml of ice-cold wash buffer and quantified by standard liquid scintillation counting techniques.

RESULTS

Phorbol esters reduce group II mGluR function at the medial perforant path synapse

Although previous studies have shown that group II mGluR function can be disrupted at corticostriatal synapses by the application of phorbol esters, it is not known whether this is a more general phenomenon or is restricted to these corticostriatal synapses. We and others previously reported that a group II mGluR presynaptically reduces transmission at the MPP synapse (Brown and Reymann, 1995; Ugolini and Bordi, 1995; Macek et al., 1996; Dietrich et al., 1997). Therefore, we determined the effect of activation of PKC by phorbol esters on group II mGluR function at the MPP–dentate gyrus synapse. Consistent with previous reports, the group II mGluR agonist DCG-IV (3 μM) induced a significant reduction of fEPSPs (Fig. 1). The PKC activator PDBu (10 μM) induced an increase in fEPSP slope at the MPP–dentate gyrus synapse, which typically stabilized after 15–20 min. After the fEPSP stabilized, the effect of DCG-IV was measured. Interestingly, PDBu markedly attenuated the inhibitory effect of

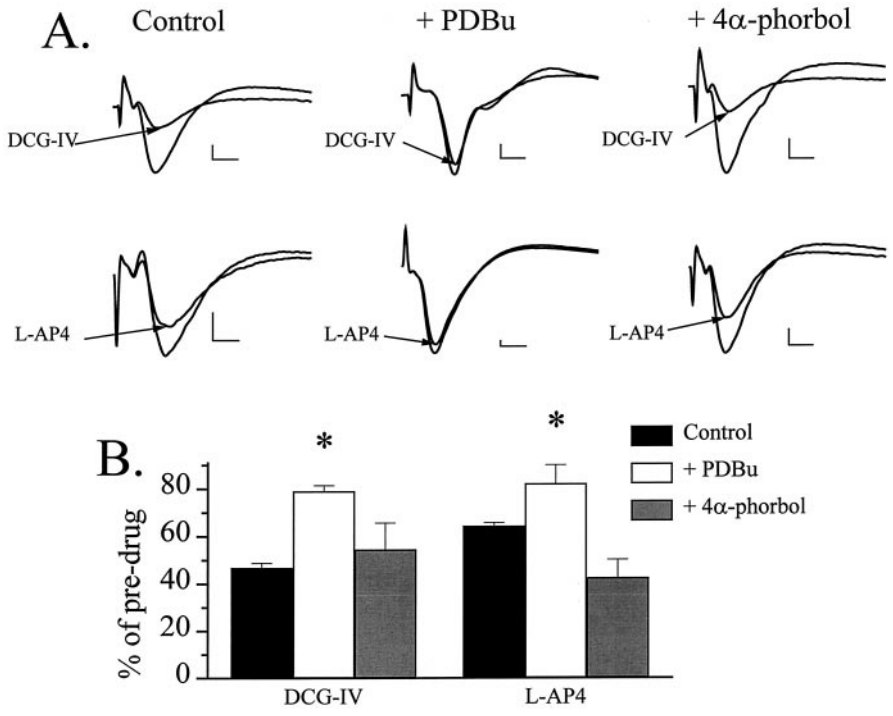

Figure 1. Activation of PKC reduces presynaptic group II and group III mGluR-induced inhibition of transmission at the MPP synapse. *A*, Representative fEPSP traces depicting the inhibitory effect of DCG-IV (3 μM) or L-AP4 (3 mM) on transmission at the MPP synapse after 20 min of exposure to vehicle (DMSO control), PDBu (10 μM), and 4α-phorbol (10 μM). These and subsequent traces are each from different slices. Calibration: 0.2 mV, 2.5 msec. *B*, Summary graph of mean ± SEM data showing the effect of phorbol esters on the inhibitory effects of DCG-IV and L-AP4 at the MPP synapse. n = 3–5 for each experiment; one-way ANOVA; $^*p < 0.05$.

DCG-IV at the MPP synapse (Fig. 1). To control for potential nonspecific actions of phorbol esters, we determined the effect of DCG-IV at the MPP after exposure to a non-PKC-activating phorbol ester, 4α-phorbol. A 20 min application of 4α-phorbol (10 μM) had no effect on the inhibition by DCG-IV (3 μM) at the MPP synapse and was not significantly different from control (Fig. 1).

Phorbol esters reduce presynaptic group III mGluR function at three major excitatory hippocampal synapses

The studies described above, coupled with a recent report at the mossy fiber–CA3 synapse (Kamiya and Yamamoto, 1997), suggest that PKC activation can reduce presynaptic function of group II mGluRs at multiple excitatory synapses. However, there is evidence that both group II and group III mGluRs reduce transmission at each of the three major excitatory hippocampal synapses, depending on stage of development, and different mGluR subtypes mediate this inhibitory function at different synapses (Koerner and Cotman, 1981; Lanthorn et al., 1984; Baskys and Malenka, 1991; Gereau and Conn, 1995; Vignes et al., 1995; Bradley et al., 1996; Bushell et al., 1996; Kamiya et al., 1996; Macek et al., 1996; Shigemoto et al., 1997). Thus, we also determined the effect of PKC activators at three synapses at which group III mGluRs serve to regulate glutamate release in the adult rat hippocampus. High (millimolar) concentrations of the group III mGluR agonist L-AP4 reduce transmission at the MPP–dentate gyrus synapse and the SC–CA1 synapse. Pharmacological characterizations of these responses (Koerner and Cotman, 1981; Baskys and Malenka, 1991; Gereau and Conn, 1995; Johansen et al., 1995; Manzoni and Bockaert, 1995; Macek et al., 1996; Dietrich et al., 1997), combined with immunocytochemical studies of group III mGluR distribution (Bradley et al., 1996; Shigemoto et al., 1997), suggest that these responses likely are mediated by mGluR7. Consistent with previous reports, high concentrations of L-AP4 (3 mM) significantly reduced fEPSP slope at the MPP synapse (Fig. 1). In contrast, L-AP4 had little effect on transmission at this synapse when the L-AP4 application was preceded by the application of PDBu (10 μM) (Fig. 1). The inactive phorbol ester 4α-phorbol (10 μM) had no effect on the inhibitory action of L-AP4 at the MPP synapse. Studies at the SC–CA1 synapse yielded similar results. L-AP4 (1 mM) virtually abolished transmission at the SC–CA1 synapse under control conditions (Fig. 2). As at the MPP synapse, PDBu (10 μM) induced a significant increase in fEPSP slope, which typically stabilized after 15–20 min. After the fEPSP stabilized, the effect of L-AP4 was measured. PDBu reduced the inhibitory effect of L-AP4 at the SC–CA1 synapse. 4α-phorbol (10 μM) had no effect on the inhibitory action of L-AP4 at the SC–CA1 synapse and was not significantly different from control (Fig. 2).

L-AP4 also reduces transmission at the LPP–dentate gyrus synapse (Koerner and Cotman, 1981; Kahle and Cotman, 1993b; Johansen et al., 1995; Macek et al., 1996; Dietrich et al., 1997). However, in contrast to the MPP–dentate gyrus synapse, the LPP synapse is sensitive to low micromolar concentrations of L-AP4 (Koerner and Cotman, 1981; Kahle and Cotman, 1993a; Johansen et al., 1995; Macek et al., 1996; Dietrich et al., 1997). On the basis of pharmacological and immunocytochemical studies, it is believed that the group III mGluR subtype differs from the presynaptic group III mGluR at the MPP and SC–CA1 synapses (Johansen et al., 1995; Bradley et al., 1996; Macek et al., 1996; Dietrich et al., 1997; Saugstad et al., 1997; Shigemoto et al., 1997). Therefore, we also determined the effect of PKC activation on L-AP4-induced inhibition of transmission at the LPP–dentate gyrus synapse. As previously reported (Koerner and Cotman, 1981; Kahle and Cotman, 1993a; Johansen et al., 1995; Macek et al., 1996; Dietrich et al., 1997), low concentrations of L-AP4 (20 μM) reduced the fEPSP slope at the LPP synapse (Fig. 3). Subsequent application of PDBu (10 μM) induced a significant increase in fEPSP slope similar to that seen at MPP and SC–CA1 synapses. Furthermore, PDBu completely inhibited the ability of

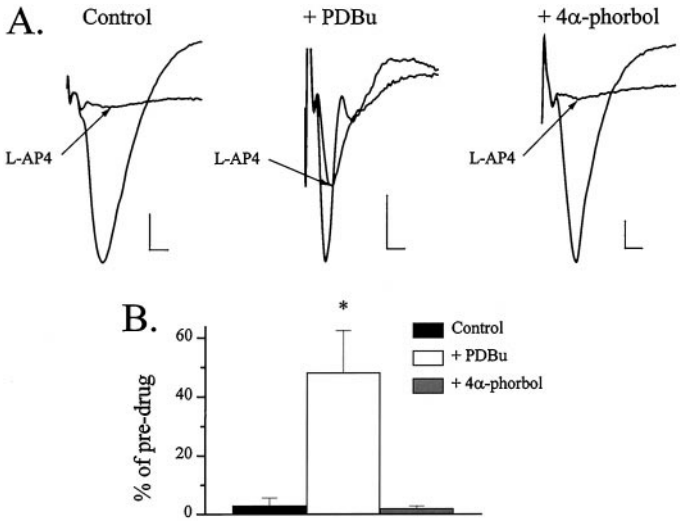

Figure 2. Activation of PKC reduces presynaptic group III mGluR-induced inhibition of transmission at the SC–CA1 synapse. *A*, Representative fEPSP traces depicting the effect of L-AP4 (1 mM) after 20 min of exposure to vehicle (DMSO control), PDBu (10 μM), or 4α-phorbol (10 μM). Calibration: 0.2 mV, 2.5 msec. *B*, Summary graph of mean ± SEM data showing the effect of phorbol esters on the inhibitory actions of L-AP4 at the SC–CA1 synapse. $n = 3–4$ for each experiment; one-way ANOVA; $^*p < 0.05$.

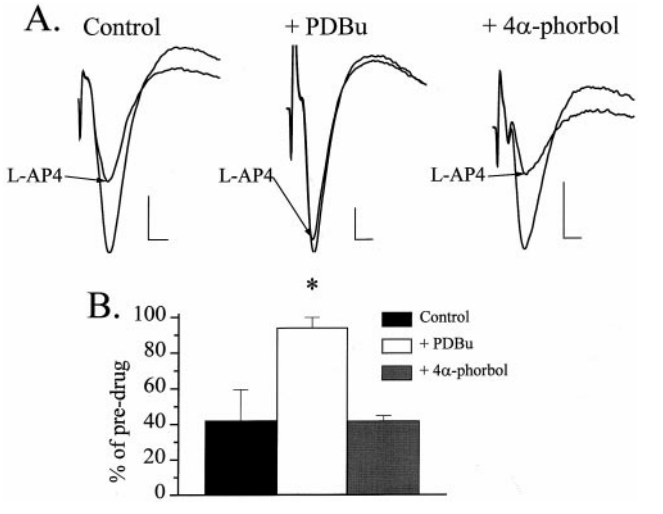

Figure 3. Activation of PKC reduces presynaptic group III mGluR-induced inhibition of transmission at the LPP synapse. *A*, Representative fEPSP traces depicting the effect of L-AP4 (20 μM) after 20 min of exposure to vehicle (DMSO control), PDBu (10 μM), or 4α-phorbol (10 μM). Calibration: 0.2 mV, 2.5 msec. *B*, Summary graph of mean ± SEM data showing the effect of phorbol esters on L-AP4-induced inhibition at the LPP synapse. $n = 3$ for each experiment; one-way ANOVA; $^*p < 0.05$.

L-AP4 (20 μM) to reduce fEPSP slope at the LPP synapse (Fig. 3). In contrast, 4α-phorbol had no significant effect on L-AP4-induced inhibition of transmission at this synapse (Fig. 3).

A_3 adenosine receptor agonists reduce group III mGluR-mediated inhibition at SC–CA1

The studies presented above, coupled with previous studies (Swartz et al., 1993; Kamiya and Yamamoto, 1997), suggest that PKC-induced inhibition of mGluR function is a relatively widespread phenomenon and that activation of PKC disrupts the presynaptic inhibitory function of multiple mGluR subtypes at multiple excitatory synapses. However, it is not yet known whether this effect can be elicited only with direct PKC activators or whether activation of receptors that are coupled to activation of phosphoinositide hydrolysis and PKC can elicit this response. Recent studies suggest that A_3 adenosine receptors couple to activation of phosphoinositide hydrolysis and are a potential site for the modulation of presynaptic inhibitory pathways via PKC activation (Abbracchio et al., 1995; Dunwiddie et al., 1997). Thus, we conducted a series of experiments to test the hypothesis that activation of A_3 adenosine receptors would induce a PKC-mediated reduction of mGluR function at the SC–CA1 synapse. As described above, 1 mM L-AP4 dramatically reduced fEPSPs in area CA1. After L-AP4 washout, the A_3 adenosine receptor agonist Cl-IB-MECA (1 μM) was applied for 20 min. Cl-IB-MECA had no significant effect on baseline transmission at the SC–CA1 synapse but significantly reduced the effect of L-AP4 (1 mM) at this synapse (Fig. 4). The effect of Cl-IB-MECA was reversible, and the response to L-AP4 was restored completely when the A_3 adenosine agonist was washed from the slice (data not shown). The response to Cl-IB-MECA was blocked completely by MRS1191 (10 μM), an A_3 adenosine-selective antagonist, suggesting that this effect is, indeed, mediated by activation of A_3 adenosine receptors (Fig. 4). Furthermore, this response was blocked by Bis (1 μM), a selective cell-permeable inhibitor of PKC (Toullec et al., 1991). As shown in Figure 5, the effect of L-AP4 in the presence of both Cl-IB-MECA and Bis was not significantly different from the response to L-AP4 alone or in the presence of vehicle controls. These data are consistent with the hypothesis that the inhibitory actions of A_3 adenosine receptor activation on group III mGluR function at the SC–CA1 synapse are mediated, at least in part, by a PKC-dependent mechanism.

Interestingly, Cl-IB-MECA (10 μM) had no effect on L-AP4- or DCG-IV-induced reduction of transmission at either the MPP or the LPP synapses (Fig. 6). Thus, although the inhibitory effect of PKC activation was present at all of the synapses that were examined, the role of A_3 adenosine receptors in eliciting this response is synapse-specific.

PKC activation disrupts group II and group III mGluR inhibition of forskolin-stimulated cAMP accumulation

As with many other receptors that are capable of regulating neurotransmitter release, group II and group III mGluRs also can couple to the inhibition of adenylyl cyclase in both expression systems and in hippocampal slices (Manzoni et al., 1992; Schoepp et al., 1992, 1995); (for review, see Conn and Pin, 1997). However, extensive studies suggest that the ability of G_i-coupled receptors to inhibit neurotransmitter release is not related to the ability of these receptors to inhibit adenylyl cyclase (Limbird, 1988; Herrero et al., 1996). Thus, these two responses likely represent two distinct signaling pathways of the same receptors. To determine whether the effect of PKC is specific to mGluR-mediated reduction in synaptic transmission or inhibits multiple responses to group II and group III mGluR function, we determined the effect of PKC activation on the inhibition of forskolin-stimulated cAMP accumulation by group II and group III mGluR agonists. As previously described, the application of either DCG-IV (10 μM) or L-AP4 (1 mM) inhibited forskolin-stimulated cAMP accumulation in hippocampal slices (Fig. 7). In the presence of PDBu (10 μM), however, the inhibitory effects of DCG-IV and L-AP4 were reduced significantly. In contrast, 4α-phorbol (10 μM) had no

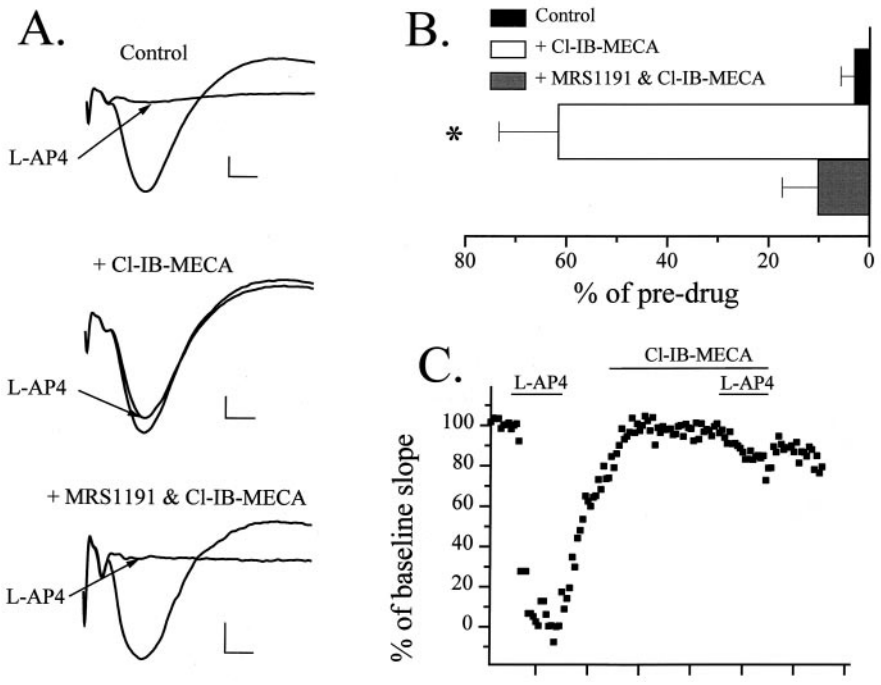

Figure 4. A_3 adenosine receptor agonists reduce group III mGluR-induced inhibition of transmission at SC–CA1 synapse. *A*, Representative fEPSP traces depicting the effect of a 20 min application of Cl-IB-MECA (1 μM) on the inhibitory actions of L-AP4 at the SC–CA1 synapse. Application of MRS1191 (10 μM) for 10 min before Cl-IB-MECA blocked the effect of Cl-IB-MECA on L-AP4-induced inhibition. Calibration: 0.2 mV, 2.5 msec. *B*, Mean ± SEM data depicting the effect of A_3 adenosine agonists and antagonists on L-AP4-induced inhibition. n = 3–5 for each experiment; one-way ANOVA; $^*p < 0.05$. *C*, Time course depicting the effect of Cl-IB-MECA on L-AP4-induced inhibition. *Axis ticks* represent 10 min intervals.

effect on the inhibition of forskolin-stimulated cAMP accumulation by DCG-IV or L-AP4 (Fig. 7).

PKC activation disrupts coupling of group II and group III mGluRs to G-proteins

The finding that PKC inhibits presynaptic mGluR function and inhibits the mGluR-induced regulation of cAMP accumulation suggests that PKC may act at a site that is proximal to the receptor or G-protein and that is common to coupling of the receptor to both of these effectors. However, it is also possible that PKC could phosphorylate downstream proteins, such as adenylyl cyclase and proteins involved in exocytosis, to inhibit mGluR effects on these effectors. To address this question, we established a system in which the receptor and G-protein complex were isolated experimentally from downstream effectors. Activation of G-protein-coupled receptors leads to a liberation of GDP from the G-protein and subsequent binding of GTP to the G-protein. If a nonhydrolyzable analog of GTP such as GTPγS is used, it is possible to measure receptor activation as a function of agonist-induced increases in GTPγS binding. This technique has been used previously to measure the coupling of other G-protein-coupled receptors to GTP-binding proteins (Akam et al., 1997; Fan et al., 1998). Both DCG-IV and L-AP4 induced a concentration-dependent increase in [^{35}S]-GTPγS binding in synaptosomal membranes prepared from rat cortex (Fig. 8). The

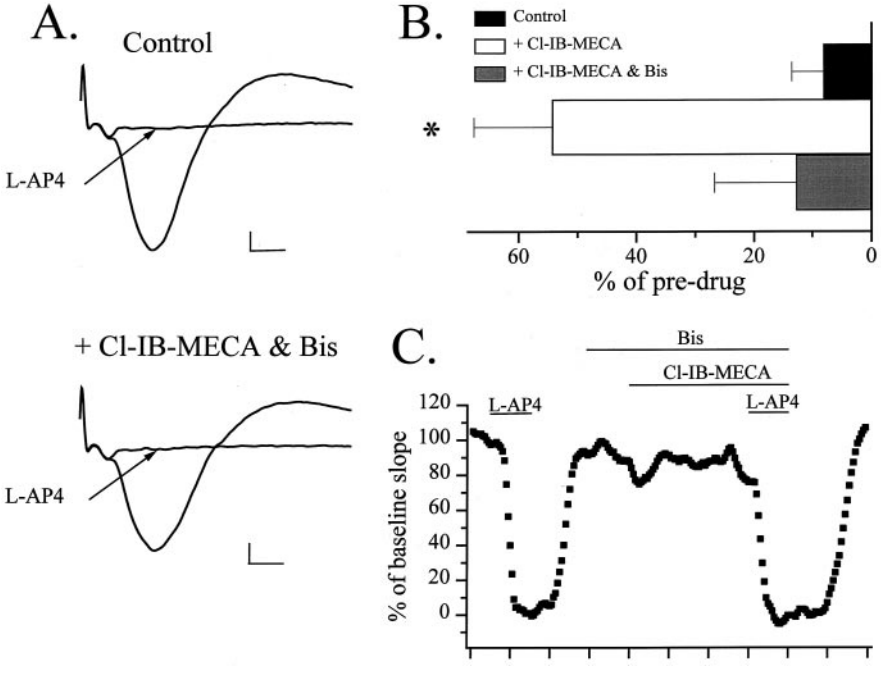

Figure 5. A_3 adenosine receptor agonists reduce L-AP4-induced inhibition of transmission at the SC–CA1 synapse by a PKC-dependent pathway. *A*, Representative fEPSP traces depicting the effect of a 10 min application of Bis (1 μM) on the reduction of L-AP4-induced (1 mM) inhibition by a 20 min application of Cl-IB-MECA (1 μm). Calibration: 0.2 mV, 2.5 msec. *B*, Mean ± SEM data showing the effect of Bis on the inhibitory actions of Cl-IB-MECA on L-AP4-induced reduction of transmission. n = 3–5 for each experiment; one-way ANOVA; $^*p < 0.05$. *C*, Time course showing that the application of Bis blocks the effect of Cl-IB-MECA on L-AP4-induced inhibition. *Axis ticks* represent 10 min intervals.

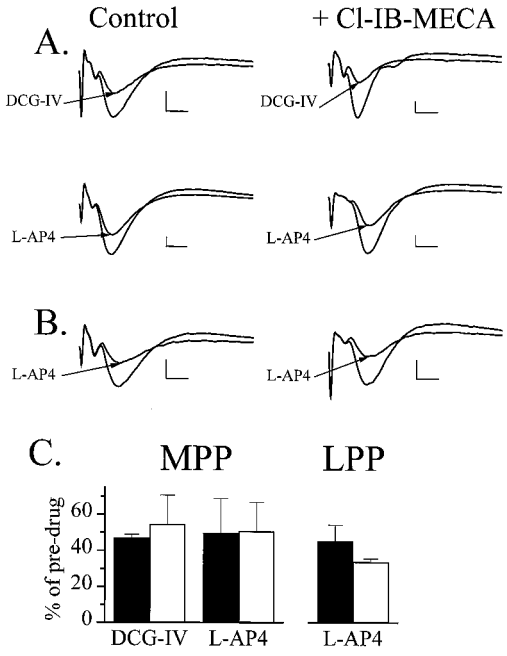

Figure 6. A_3 adenosine receptor agonists do not reduce group II or group III mGluR effects at perforant path synapses. *A*, fEPSP traces depicting the lack of effect of a 20 min application of Cl-IB-MECA (10 μM) on DCG-IV (3 μM) or on L-AP4-induced (3 mM) inhibition of transmission at the MPP synapse. *B*, fEPSP traces depicting the lack of effect of preapplication of Cl-IB-MECA (10 μM) on the inhibitory effect of L-AP4 (20 μM) at the LPP synapse. Calibration for *A, B*: 0.2 mV, 2.5 msec. *C*, Mean ± SEM data depicting the lack of effect of Cl-IB-MECA (10 μM) on mGluR inhibition at perforant path synapses. *Black bars* represent the degree of inhibition by mGluR agonists under control conditions, and *white bars* represent the degree of inhibition by mGluR agonists after a 20 min application of Cl-IB-MECA (10 μM). $n = 3–4$ for each experiment; unpaired Student's *t* test.

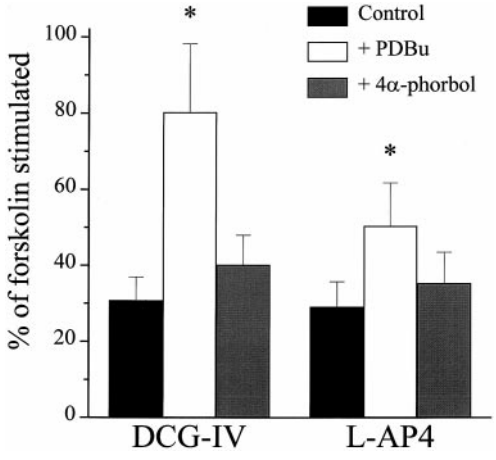

Figure 7. Phorbol esters reduce group II and group III mGluR-induced inhibition of forskolin-stimulated cAMP accumulation in hippocampus. Mean ± SEM data show the effect of PDBu (10 μM) or 4α-phorbol (10 μM) on DCG-IV-induced (10 μM) or L-AP4-induced (1 mM) inhibition of forskolin-stimulated cAMP accumulation in cross-chopped hippocampal slices. $n = 4$ experiments for each, done in triplicate; one-way ANOVA; $^*p < 0.05$.

EC_{50} for DCG-IV-induced [^{35}S]-GTPγS binding was 248 nM, and the EC_{50} for L-AP4 was 620 nM. The response to L-AP4 appeared biphasic and at higher (millimolar) concentrations induced a further increase in [^{35}S]-GTPγS binding (data not shown). We next determined the effect of PKC activation on mGluR coupling to G-proteins by determining the effects of PKC activation on [^{35}S]-GTPγS binding. Synaptosomes were prepared under conditions in which the cortical slices first were incubated for 30 min in vehicle alone, PDBu, or 4α-phorbol. The effects of DCG-IV (10 μM) and L-AP4 (30 μM) on [^{35}S]-GTPγS binding were reduced significantly in synaptosomes prepared from slices incubated in PDBu (10 μM) for 30 min before synaptosome preparation (Fig. 8). 4α-phorbol had no effect on DCG-IV or L-AP4-induced increases in [^{35}S]-GTPγS binding (Fig. 8).

DISCUSSION

Previous studies by Swartz et al. (1993) revealed that activation of PKC can reduce dramatically the presynaptic inhibitory functions of a group II mGluR at corticostriatal synapses. This finding provided important new insights into our understanding of the mechanisms of regulation of corticostriatal function and suggested that stimuli that activate presynaptic PKC had the potential of enhancing transmission at this synapse by removing the inhibitory influence of presynaptic mGluRs. However, from these studies it was not clear whether PKC-induced inhibition of mGluR function is restricted to group II mGluRs at corticostriatal synapses or is a more general phenomenon. In the present report we demonstrate that activators of PKC inhibit presynaptic mGluR function at three major synapses in the hippocampal formation. These include the LPP, MPP, and SC–CA1 synapses. Pharmacological and immunocytochemical studies suggest that mGluR7 is likely to be the group III mGluR subtype that presynaptically reduces transmission at the MPP–dentate gyrus and the SC–CA1 synapse (Koerner and Cotman, 1981; Gereau and Conn, 1995; Bradley et al., 1996; Macek et al., 1996; Shigemoto et al., 1997), but the mGluR that presynaptically reduces transmission at the LPP synapse of the adult rat is most likely a subtype other than mGluR7. Based on pharmacological and immunocytochemical studies, of the other cloned group III mGluRs, mGluR8 is the most likely candidate to mediate the presynaptic inhibitory mGluR function at the LPP synapse (Johansen et al., 1995; Macek et al., 1996; Dietrich et al., 1997; Saugstad et al., 1997; Shigemoto et al., 1997). Immunocytochemical studies suggest that mGluR2 is the most likely candidate for the group II mGluR subtype that presynaptically reduces transmission at corticostriatal and MPP–DG synapses (Neki et al., 1996; Petralia et al., 1996; Shigemoto et al., 1997). Furthermore, Kamiya and Yamamoto (1997) recently reported that PKC activators reduce group II mGluR function at the mossy fiber–CA3 synapse. Thus, PKC inhibits mGluR function at each of five major excitatory synapses that have been examined to date. Taken together, these data suggest that PKC-induced inhibition of mGluR-mediated responses is a general phenomenon and is not restricted to the regulation of group II mGluRs at corticostriatal synapses.

Inhibition of mGluR function by PKC could play a critical role in fine-tuning mGluR function and thereby regulating transmission at glutamatergic synapses in both normal physiological conditions and in various pathological conditions. For instance, activation of PKC has been shown to play an important role in the induction of hippocampal long-term potentiation and some other forms of synaptic plasticity (for review, see Conn and Sweatt, 1994). Although inhibition of presynaptic mGluR function is not likely to be the only role of PKC in induction of LTP, this effect could contribute to the induction of LTP by enhancing glutamatergic transmission during periods of high-frequency synaptic

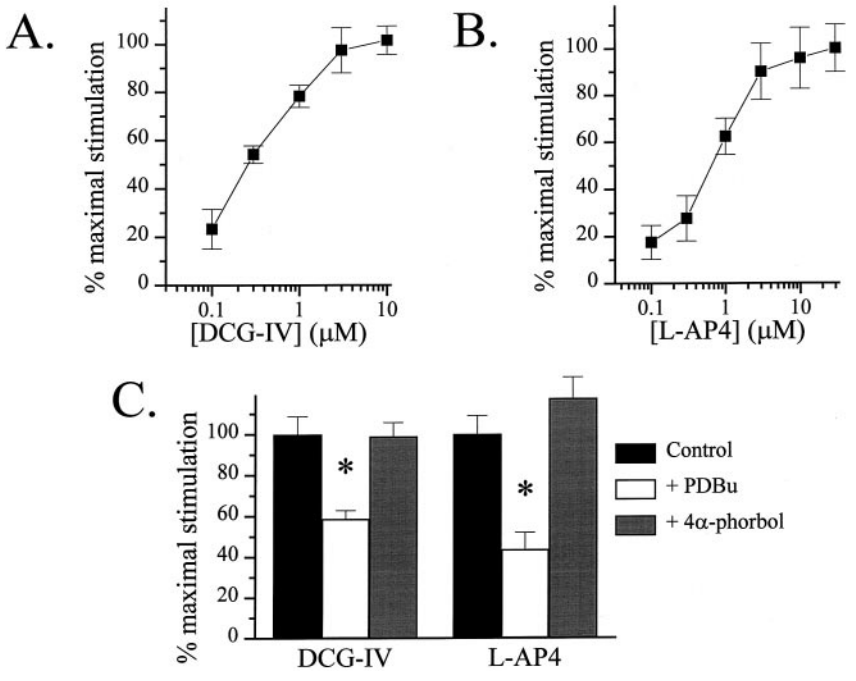

Figure 8. Phorbol esters disrupt the coupling of mGluRs to G-proteins in cortical synaptosomes. Concentration–response curves show the effects ± SEM of increasing concentrations of DCG-IV (*A*) ($n = 3$ for each point) and L-AP4 (*B*) ($n = 4$ for each point) on GTPγS binding. *C*, Bar graph of mean ± SEM data showing the percentage of GTPγS binding stimulated by DCG-IV (10 μM) or L-AP4 (30 μM) in synaptosomes preincubated in DMSO (*Control*), PDBu (10 μM), or 4α-phorbol (10 μM). $n = 3$ for each experiment; one-way ANOVA; $^*p < 0.05$.

activity. Effects of PKC on mGluR function also could enhance glutamate-induced excitotoxicity. For instance, activation of group II and group III mGluRs can have a neuroprotective action (Bruno et al., 1994, 1995, 1996), an effect that may be partially attributable to the inhibitory effects of these receptors on glutamate release. Thus, inhibition of group II and group III mGluR function by PKC could diminish this neuroprotective action and enhance excitotoxicity.

Another important implication of these findings is that selective agonists or antagonists of receptors that are coupled to activation of PKC could provide novel therapeutic targets for the development of drugs that could be used to regulate mGluR function. For instance, agonists of presynaptic receptors that are coupled to activation of PKC could provide a mechanism for inducing relatively subtle increases in glutamatergic transmission in a manner that would be dependent on the activity of glutamatergic neurons and may induce less toxicity than would direct-acting agonists of postsynaptic receptors. Such agents could be useful as cognitive-enhancing agents for the treatment of Alzheimer's disease and related disorders or for the treatment of disorders such as schizophrenia, which are thought to involve a hypofunction of glutamatergic transmission (for review, see Olney and Farber, 1995; Hirsch et al., 1997). Likewise, selective antagonists of receptors involved in inhibiting mGluR function could lead to a subtle decrease in glutamatergic transmission and may offer some neuroprotection in neurodegenerative disorders involving excitotoxicity.

Despite these exciting possibilities, all of the previous studies used phorbol esters to activate PKC, and it was not known whether agonists of receptors that are coupled to the activation of phosphoinositide hydrolysis (and PKC) could elicit this effect. The present finding that A_3 adenosine receptor activation disrupts mGluR function provides a major advance in establishing a role for more physiologically relevant stimuli in eliciting this effect. However, it was interesting to find that the effect of A_3 adenosine receptor activation was seen only at the SC–CA1 synapse, although PKC activation reduced presynaptic mGluR function at all of the synapses that were examined. Thus, although the role of PKC in eliciting this response may be widespread, there is likely to be synapse specificity with regard to the neuromodulatory agents involved in activating PKC.

Although the effect of A_3 adenosine receptor activation appears to be synapse-specific, it may not be entirely specific for the mGluR-mediated reduction of synaptic transmission relative to the effects of other modulatory agents that reduce glutamate release at the SC–CA1 synapse. For instance, Dunwiddie et al. (1997) recently reported that activation of A_3 adenosine receptors can regulate responses to A_1 adenosine receptor activation. However, activation of A_3 adenosine receptors has no effect on the inhibitory effects of $GABA_B$ or muscarinic acetylcholine receptor activation on glutamate release at the SC–CA1 synapse. Thus, whereas multiple modulators can reduce transmission at the SC–CA1 synapse, the effect of A_3 adenosine receptor activation may be selective for mGluR and A_1 adenosine receptor-mediated responses.

In light of the inhibitory effects of A_3 adenosine receptor agonists shown here, it is interesting to note that previous studies demonstrated that the application of A_3 adenosine agonists increased the neuronal damage induced by ischemic insult (von Lubitz et al., 1994). The mechanism by which this occurs is unknown; however, it is tempting to speculate that activation of A_3 adenosine receptors disrupts the inhibitory actions of mGluR and A_1 adenosine receptor activation on glutamate release and thereby exacerbates glutamate release and neuronal damage during the ischemic insult.

The present studies also provide a step forward in developing an understanding of the potential cellular mechanisms by which PKC could inhibit presynaptic mGluR function. One mechanism that has been proposed to underlie the PKC-induced suppression of some other presynaptic receptors that inhibit neurotransmitter release is direct phosphorylation of voltage-gated calcium channel subunits and the inhibition of receptor-mediated inhibition of calcium currents (Zamponi et al., 1997). However, it is unlikely that this mechanism could explain fully the effects of PKC on

mGluR function. For instance, whereas mGluR-induced reduction of glutamate release is likely to be mediated by the inhibition of calcium currents at some synapses, such as the calyx of Held (Takahashi et al., 1996), mGluR7-induced reduction of transmission at the SC–CA1 synapse is not likely to be mediated by the inhibition of calcium currents (Gereau and Conn, 1995). Similar results for a group II mGluR have been reported at excitatory synapses formed by mitral cells of olfactory bulb (Schoppa and Westbrook, 1997). Furthermore, we found that PKC activators also inhibit the coupling of both group II and group III mGluRs to the inhibition of forskolin-stimulated adenylyl cyclase, a response that is unrelated to mGluR-induced inhibition of calcium currents (Limbird, 1988; Ikeda, 1996). These findings prompted us to test the hypothesis that PKC acts at a site upstream from the calcium channels or other effector proteins. Consistent with this, we found that PKC activation inhibits the ability of group II and group III mGluRs to increase [^{35}S]-GTPγS binding to synaptosomal membranes. These findings suggest that PKC-induced inhibition of group II and group III mGluR signaling could be mediated at least partially by the inhibition of coupling of the receptors to GTP-binding proteins. This could occur by phosphorylation of a site on either the receptor or the G-protein. However, it is important to keep in mind that these biochemical studies were performed in cross-chopped slices and cortical synaptosomes. Thus, these studies represent the average effects of PKC activators on multiple mGluR subtypes in both neurons and glia. In contrast, the electrophysiology studies reveal effects of PKC activation on mGluRs at specific synapses. Thus, it is possible that other mechanisms also are involved in inhibiting mGluR function at specific presynaptic sites.

Interestingly, previous studies have demonstrated that PKC also is involved in agonist-induced desensitization of mGluR5, a group I mGluR (Alaluf et al., 1995; Kawabata et al., 1996; Gereau and Heinemann, 1998). Biochemical and molecular studies suggest that mGluR5 desensitization is mediated by direct phosphorylation of the receptor by PKC. Thus, PKC may have similar actions on all three of the major mGluR subgroups. In future studies it will be interesting to determine whether PKC directly phosphorylates group II and group III mGluRs and to determine whether phosphorylation of the receptors inhibits their ability to couple to GTP-binding proteins.

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