Differential Actions of PKA and PKC in the Regulation of Glutamate Release by Group III mGluRs in the Entorhinal Cortex

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Evans, D. Ieuau, Roland S. G. Jones, and Gavin Woodhall. Differential actions of PKA and PKC in the regulation of glutamate release by group III mGluRs in the entorhinal cortex. J Neurophysiol 85: 571–579, 2001. In a previous study we showed that activation of a presynaptically located metabotropic glutamate receptor (mGluR) with pharmacological properties of mGluR4a causes a facilitation of glutamate release in layer V of the rat entorhinal cortex (EC) in vitro. In the present study we have begun to investigate the intracellular coupling linking the receptor to transmitter release. We recorded spontaneous α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor–mediated excitatory postsynaptic currents (EPSCs) in the whole cell configuration of the patch-clamp technique, from visually identified neurons in layer V. Bath application of the protein kinase A (PKA) activator, forskolin, resulted in a marked facilitation of EPSC frequency, similar to that seen with the mGluR4a specific agonist, ACPT-1. Preincubation of slices with the PKA inhibitor H-89 abolished the effect of ACPT-1, as did preincubation with the adenylate cyclase inhibitor, SQ22536. Activation of protein kinase C (PKC) using phorbol 12 myristate 13-acetate (PMA) did not affect sEPSC frequency; however, it did abolish the facilitatory effect of ACPT-1 on glutamate release. A robust enhancement of EPSC frequency was seen in response to bath application of the specific PKC inhibitor, GF 109203X. Both H-89 and the group III mGluR antagonist (RS)-α-cyclopropyl-4-phosphonophenylglycine (CPPG) abolished the effects of GF 109203X. These data suggest that in layer V of the EC, presynaptic group III mGluRs facilitate release via a positive coupling to adenylate cyclase and subsequent activation of PKA. We have also demonstrated that the PKC system tonically depresses transmitter release onto layer V cells of the EC and that an interaction between mGluR4a, PKA, and PKC may exist at these synapses.

INTRODUCTION

Neurotransmitter release is governed by many complex and interacting factors in the presynaptic terminal. One powerful means of controlling release is via receptors located on the terminals, which can be acted on either by the same transmitter released from those terminals (autoreceptors), or by a different transmitter (heteroreceptors). There are an increasing number of examples of both types of receptor at central synapses, and these can be either ionotropic or metabotropic in nature (see Manahan-Vaughan et al. 1999; Miller 1998; Starke et al. 1989). In this laboratory we have been studying the physiology and pharmacology of synaptic function in the entorhinal cortex (EC), particularly with respect to epileptogenesis. One of the main focuses of this work is to examine how transmitter release at glutamate and GABA synapses is modulated by presynaptic receptors.

We have previously shown that glutamate release is tonically facilitated via presynaptic N-methyl-D-aspartate (NMDA) autoreceptors in both layer II and layer V of the EC (Berretta and Jones 1996; Woodhall and Jones 1999). Current studies have also shown that spontaneous GABA release onto layer V neurons is inhibited by presynaptic GABA_3 autoreceptors, a control that may also be tonically active (Wood and Jones 1999; Wood et al. 1999). In addition, we have recently examined the role of presynaptic metabotropic glutamate receptors (mGluRs). The mGluRs are a group of G-protein–coupled receptors that have been shown to modulate synaptic transmission via intracellular signaling pathways. The mGluRs have been subdivided into three groups based on their amino acid sequence homology, pharmacology, and intracellular coupling (for review see Conn and Pin 1997). Group III mGluRs (mGluRs 4, 6, 7, and 8) have been reported to depress glutamatergic transmission in a number of brain areas (Baskys and Malenka 1991; Davies and Watkins 1982; Dube and Marshall 1997; Forsythe and Clements 1990; Jin and Daw 1998; Trombly and Westbrook 1992), and this has been suggested to be through a negative coupling to adenylate cyclase (Okamoto et al. 1994; Saugstad et al. 1997; Tanabe et al. 1993), leading to inhibition of calcium currents in presynaptic terminals (Glaum and Miller 1995; Takahashi et al. 1996). Another report has implicated G-protein βγ sub-units in group III mGluR-mediated inhibition of glutamate release (O’Connor et al. 1999).

In common with reports from other brain areas, we found that activation of group III mGluRs with the agonist, (S)-(+)2-amino-4-phosphonobutyric acid (l-AP4) reduced glutamate release from terminals in layer II of the EC (Evans et al. 2000). However, in complete contrast, the same agonist caused a powerful facilitation of glutamate release in layer V (Evans et al. 2000). This may suggest that the response in layer V is due to a different receptor subtype or a receptor with a different intracellular coupling system to those previously described. Since further pharmacological analysis confirmed that the facilitation of release was mediated by group III receptors, and indicated that it was likely to be the result of activation of mGluR4a, we think the latter explanation to be the most likely.

In the present study we have begun to define intracellular
signaling mechanisms that may underlie the facilitatory effect of mGluR4a on glutamate release by looking at the effect of activation or inhibition of protein kinases. Some of this work has been presented in abstract form (Evans et al. 1999).

METHODS

Hippocampal-EC slices were prepared from male Wistar rats (50–110 g) as previously described (Jones and Heinemann 1988). In brief, rats were anesthetized with an intramuscular injection of ketamine (120 mg/kg) plus xylazine (8 mg/kg) and decapitated. The brain was rapidly removed and immersed in oxygenated artificial cerebrospinal fluid (ACSF) chilled to 4°C. Slices (450 μm) were cut using a vibroslice (Campden Instruments) and stored in ACSF continuously bubbled with 95% O_2-5% CO_2 maintained at room temperature. Following a recovery period of at least 1 h, individual slices were transferred to a recording chamber mounted on the stage of an Olympus upright microscope (BX50WI). The chamber was continuously perfused with oxygenated ACSF at 30–32°C at a flow rate of approximately 2 ml/min. The ACSF contained the following (in mM): 126 NaCl, 4 KCl, 1.25 NaH_2 PO_4, 24 NaHCO_3, 2 MgSO_4, 2.5 CaCl_2, and 10 d-glucose. The solution was continuously bubbled with 95% O_2-5% CO_2 to maintain a pH of 7.4. Neurons were visualized using differential interference contrast optics and an infrared video camera.

Patch-clamp electrodes were pulled from borosilicate glass (1.5 mm OD, 0.69 ID; Clark Electromedical) and had open tip resistances of 4–5 MΩ. They were filled with a solution containing the following (in mM): 130 Cs-methanesulphonate, 10 HEPES, 5 QX-314, 0.5 EGTA, 1 NaCl, 0.34 CaCl_2, 1 MK801, 4 ATP, and 0.4 GTP. The solution was adjusted to 290 mOsmol with sucrose and to pH 7.3 with CsOH. Whole cell voltage-clamp recordings were made using an Axopatch 200B amplifier (Axon Instruments), and neurons were clamped at −60 mV. Signals were filtered at 2 kHz and digitized at 20 kHz. Access resistance was monitored at regular intervals and cells rejected if this parameter changed by more than 15%. Data were recorded directly to computer hard disk using Axoscope software (Axon Instruments). Under these experimental conditions, EC neurons within layer V exhibited excitatory postsynaptic currents (EPSCs), mediated by spontaneous release of glutamate acting at layer V exhibited excitatory postsynaptic currents (EPSCs), mediated by spontaneous release of glutamate acting at

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All salts used in preparation of ACSF were Analar grade and purchased from Merck/BDH. Drugs used were (1S,3R,4S)-1-amino-cyclopentane-1,2,4-tricarboxylic acid (ACPT-1), phorbol 12 myristate 13-acetate (PMA), forskolin, and 2-[1-dimethylaminopropyl]-5-isouquinolinesulphonamide HCl (H-89), 4z,9e,12β,13α,20-pentahydroxytiglia-1,6-dien-3-one (4z-phorbol), and 7β-acetoxy-6β-hydroxy-8,13-epoxy-labd-14-en-11-one (1,9-dideoxyforskolin) were obtained from Sigma, and dizocilpine maleate (MK801) and (9-tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536) from RBI. Unless otherwise stated, all drugs were applied by inclusion in the bath perfusion medium.

RESULTS

The results reported here are based on recordings obtained from 87 neurons in layer V. With MK-801 (1 mM) in the patch pipette solution to block postsynaptic NMDA receptors, spontaneous AMPA receptor–mediated excitatory postsynaptic currents (sEPSCs) were recorded as inward currents in neurons voltage clamped at −60 mV. sEPSCs had a mean frequency of 2.4 Hz.

Forskolin mimics activation of presynaptic mGluRs

We have previously shown that bath application of the specific group III mGluR agonist, 1-AP4 and ACPT-1, resulted in a facilitation of both spontaneous (Fig. 1) and activity-independent glutamate release onto layer V neurons, and that the receptors responsible were located presynaptically (Evans et al. 2000). At the concentration used (20 μM), ACPT-1 has been reported to act specifically at mGluR4a (Acher et al. 1997). Group III mGluRs have been shown to couple to adenylate cyclase (AC) and cAMP (Tanabe et al. 1993), so our first approach was to determine whether cAMP-dependent protein kinase A (PKA) could also modulate glutamate release onto layer V neurons. Bath application of the

![Diagram](Image)

FIG. 1. mGluR4a-induced increase in the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) recorded from layer V neurons. A: consecutive whole cell voltage-clamp records from a typical cell before, and during, bath application of the specific mGluR4a agonist, (1S,3R,4S)-1-amino-cyclopentane-1,2,4-tricarboxylic acid (ACPT-1; 20 μM). A clear increase in frequency was observed with ACPT-1. B: cumulative probability plot of inter-event-interval (IEI) under control conditions and during bath application of ACPT-1. The leftward shift in the presence of ACPT-1 indicates that frequency was increased. Pooled data from 11 cells.
Forskolin facilitates sEPSC frequency but not amplitude in layer V of the entorhinal cortex (EC).

**TABLE 1. Summary of EPSC kinetics under various conditions**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Rise Time</th>
<th>Decay Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.94 ± 0.15</td>
<td>4.49 ± 0.09</td>
</tr>
<tr>
<td>Forskolin</td>
<td>2.05 ± 0.11</td>
<td>5.80 ± 0.10</td>
</tr>
<tr>
<td>Control</td>
<td>1.91 ± 0.12</td>
<td>4.35 ± 0.50</td>
</tr>
<tr>
<td>GF 109203X</td>
<td>1.90 ± 0.12</td>
<td>4.43 ± 0.48</td>
</tr>
</tbody>
</table>

Values are means ± SE.

1,9-Dideoxyforskolin does not affect EPSC frequency

To control for nonspecific effects of forskolin, we used the forskolin analogue 1,9-dideoxyforskolin (50 μM), which does not activate adenylate cyclase (Seamon et al. 1983). In five neurons tested, the mean frequency of events was 2.17 ± 0.07 Hz under control conditions and remained unchanged at 1.67 ± 0.04 Hz on bath application of 1,9-dideoxyforskolin (P > 0.05). These data suggest that the enhancement of EPSC frequency seen on application of forskolin was indeed due to activation of PKA.

**FIG. 2.** Forskolin facilitates sEPSC frequency but not amplitude in layer V of the entorhinal cortex (EC). A: raw data recorded from a typical cell under control conditions and during bath application of forskolin (100 μM). B: cumulative probability plot of IEIs of sEPSCs under control (con) conditions and during bath application of forskolin (FSK). Forskolin clearly induces a leftward shift in the distribution of EPSCs, illustrating an increase in frequency. C: averaged EPSCs from a typical cell under control conditions and on bath application of forskolin. Forskolin induces a slight slowing of the decay time (see Table 1). D: amplitude distribution plot (bin size 2 pA). There is no change in the distribution of EPSC amplitudes on application of forskolin. Pooled data from 12 cells.

**FIG. 3.** H-89 prevents the effects of ACPT-1. In the presence of the specific PKA inhibitor H-89 (100 μM), the effects of ACPT-1 are abolished. A: raw data from a typical cell before and during bath application of ACPT-1 (20 μM) in the presence of H-89. B: cumulative probability plot of IEIs. Pooled data from 7 cells.
compare directly the frequency of sEPSCs before and during H-89 application. However, in all cells, the frequency of sEPSCs recorded in slices incubated in H-89 was very similar to control values obtained in layer V neurons in previous experiments.

**SQ 22536 prevents the facilitatory effect of ACPT-1**

Modulation of adenylate cyclase activity would be a prerequisite in a potential transduction pathway between mGluR4a and PKA. Therefore, we investigated the effect of blocking adenylate cyclase with the specific adenylate cyclase inhibitor, SQ22536 (50 μM) (Fabbri et al. 1991) on the response to ACPT-1. In seven neurons, in slices preincubated (30 min) with SQ22536, the facilitation of release by ACPT-1 was abolished. Figure 4A shows raw data traces from one neuron. Mean frequency was 2.25 ± 0.09 Hz in SQ22536 alone, and 1.78 ± 0.10 Hz in the presence of ACPT-1. The cumulative probability plots in Fig. 4B show pooled data from seven neurons, illustrating that there is no difference in IEI distribution under control conditions, and during application of ACPT-1, when SQ22536 was present (P > 0.1, KS). Again, although it was not possible to compare directly the frequency of sEPSCs before and after application of SQ22536, the mean frequency (2.25 ± 0.09 Hz) was similar to that observed under control conditions in other experiments.

**Protein kinase C (PKC) activation does not facilitate release**

Thus the data strongly indicated that PKA was involved in the facilitatory response to ACPT-1 at synapses in layer V. A second serine/threonine kinase, PKC, has been shown to have effects at synapses at which PKA is also modulatory (Capogna et al. 1995; Gubitz et al. 1996; Malenka et al. 1987). To investigate whether activation of the PKC system could also modulate the release of glutamate onto layer V cells, we used the diacylglycerol (DAG) mimetic phorbol ester, PMA. In six neurons, application of PMA (500 nM) did not result in any significant change in sEPSC frequency. Figure 5A shows raw data traces from one neuron. Mean sEPSC frequency was 4.80 ± 0.09 Hz under control conditions, and 5.03 ± 0.09 Hz (P > 0.5, KS) on bath application of PMA. The cumulative probability plots in Fig. 5B show pooled data from six neurons, illustrating that there is no difference in IEI distribution under control conditions, and during application of PMA. We did not observe the marked enhancement of EPSC frequency seen in other preparations (e.g., Carrol et al. 1998), in any of our cells, even at high concentrations of PMA. In nine neurons, bath application of PMA (10 μM) resulted in a very small decrease in sEPSC frequency from 2.20 ± 0.08 Hz to 1.85 ± 0.07 Hz (P < 0.01, KS). Figure 6A shows raw data traces from one neuron. The cumulative probability plots in Fig. 6B show pooled data from nine neurons, illustrating that there is a rightward shift in IEI distribution during application of PMA, reflecting a decrease in sEPSC frequency. As a negative control, we also bath applied the biologically inactive analogue, 4-α phorbol (10 μM), which had no effect on sEPSC frequency (control 2.43 ± 0.07 Hz, 4-α phorbol 2.50 ± 0.06 Hz, n = 5, P > 0.05, KS).

When ACPT-1 was applied after perfusion with PMA (data not shown), it failed to cause a change in mean sEPSC frequency (control in PMA, 2.62 ± 0.06 Hz; ACPT-1, 2.06 ±
0.04 Hz, \( n = 5 \), \( P < 0.05 \), KS), suggesting a potential inhibitory interaction between PKC and the group III mGluR or a downstream target. We examined this possibility further using the specific PKC inhibitor, GF 109203X (Falet and Rendu 1994).

GF 109203X facilitates glutamate release

In 13 layer V neurons, we found that inhibition of PKC resulted in a reliable enhancement of transmitter release. Bath application of GF 109203X (500 nM) caused a clear increase in sEPSC frequency. The mean frequency was 2.79 ± 0.03 Hz before and 3.46 ± 0.04 Hz during application of GF 109203X, which suggested that PKC was tonically active in suppressing activity at these synapses. Figure 7A shows sample raw traces from one neuron. Figure 7B is the cumulative probability plot showing a shift to the right, indicative of a significant \( (P < 0.001, \) KS) increase in frequency in the presence of GF 109203X. Analysis of the kinetics of sEPSCs in the presence of GF 109203X revealed no significant change in rise or decay times (Table 1), consistent with a presynaptic locus.

H-89 abolishes the effects of GF 109203X

If our hypothesis that PKC exerts tonic inhibition of glutamate release through the group III mGluR pathway is correct, then inhibition of AC or PKA activity might be expected to prevent the effect of GF 109203X and the subsequent “release” of these elements from inhibition. After 30 min incubation with the specific PKA inhibitor, H-89 (20 \( \mu \)M), subsequent bath application of the PKC inhibitor, GF 109203X, even at a relatively high concentration of 100 \( \mu \)M, no longer resulted in a facilitation of glutamate release. Figure 8A shows records from one cell showing that there was no increase in EPSC frequency when GF 109203X was applied in the presence of H-89. The mean EPSC frequency was 2.26 ± 0.10 Hz before and 2.25 ± 0.09 Hz during the application of GF 109203X. Figure 8B shows cumulative probability plots indicating that there was no detectable change in IEI distribution (pooled data from 5 cells, \( P > 0.5 \)).

Facilitatory effects of forskolin and GF 109203X are additive

It seems clear that blockade of PKA prevents the facilitatory effect of PKC inhibition on glutamate release. We reasoned that if the effects of PKC inhibition are mediated via PKA and that PKC inhibits a receptor pathway that would otherwise be tonically active, then we might predict two testable hypotheses: 1) that the effects of PKA activation would be occluded to some extent by prior inhibition of PKC and 2) that effects of PKC inhibition would be abolished by application of the mGlur4a antagonist CPPG. We attempted to occlude the effect of forskolin by prior application of GF 109203X. We applied forskolin (10 \( \mu \)M) after preequilibration with GF 109203X (500 nM). Bath application of the PKA activator forskolin resulted in an additional increase in sEPSC frequency above that observed with GF 109203X alone. Mean sEPSC frequency was 4.42 ± 0.09 Hz.
in the presence of GF 109203X and 6.23 ± 0.15 Hz after application of forskolin. Figure 9A shows traces from one neuron, and Fig. 9B shows IEI distributions where GF 109203X was applied alone or together with forskolin. There was clearly an increase in sEPSC frequency (P < 0.001, pooled data from 5 cells). Thus while blockade of PKA prevented the facilitatory effect of PKC inhibition on glutamate release, blockade of PKC did not prevent the facilitatory effect of PKA activation with forskolin. When these experiments were performed using a much larger concentration of GF 109203X (100 μM), we observed complete occlusion of the effect of forskolin (n = 5, data not shown). However, we feel that this result cannot be relied on because of potential non-specific effects of GF 109203X at this concentration, and the fact that glutamate release may already be maximal prior to forskolin application.

**CPPG reduces sEPSC frequency in the presence of GF 109203X**

The data described so far suggest that the increased sEPSC frequency seen after blockade of PKC by GF 109203X was related to disinhibition of mGluR4a. We tested this scenario directly, by applying the group III mGluR antagonist CPPG after sEPSC frequency had been enhanced by prior application of GF 109203X. In six cells tested, bath application of GF 109203X resulted in a facilitation of mean sEPSC frequency from 2.42 ± 0.08 Hz under control conditions to 3.75 ± 0.10 Hz on application of GF 109203X (P < 0.01). In the same cells, subsequent bath application of the specific group III mGluR antagonist CPPG (10 μM) in the presence of GF 109203X resulted in return of mean sEPSC frequency to a level that was statistically indistinguishable from control (3.04 ± 0.09 Hz, P > 0.01 compared with predrug control). Figure 10A shows records from one cell showing that there was a reduction in EPSC frequency when CPPG was applied in the presence of GF 109203X. Figure 10B shows the cumulative probability plots (pooled data from 6 neurons). We have previously reported that bath application of CPPG alone does not affect mean sEPSC frequency in layer V of the EC (Evans et al. 2000). Since the antagonist (CPPG) only reduces sEPSC frequency when PKC is inhibited, these data would seem to support our hypothesis that mGluR4a is under a tonic inhibitory influence by PKC, and that blockade of PKC enhances spontaneous neurotransmission via release of mGluR4a and/or PKA from this inhibitory influence.

**DISCUSSION**

The principal finding of this study was that the increase in sEPSC frequency induced by the specific mGluR4a agonist, ACPT-1, was mimicked by modulation of protein kinase activity. Activation of PKA enhanced glutamate release, whereas activation of PKC had little effect. By contrast, inhibition of PKC enhanced glutamate release, and further experiments suggested that mGluR4a/PKA and PKC interact at synapses onto layer V neurons.

PKA activation has been reported to potentiate excitatory transmitter release in the hippocampus (Chavez-Noriega and Stevens 1994; Hopkins and Johnston 1988; Malenka et al. 1986a, 1987; Weisskopf et al. 1994). In layer V of the EC, the
specific PKA activator forskolin caused a rapid increase in sEPSC frequency. The facilitation of release was similar to that we observed in response to ACPT-1 and raised the possibility that mGluR4a, present at synapses in layer V of the EC, may be positively coupled to activation of the cAMP cascade and thus to activation of PKA. This was strongly supported by the observation that the PKA inhibitor H-89, abolished the effects of ACPT-1.

To confirm that mGluR4a activation was coupled via the cAMP cascade to PKA, we blocked the signaling system upstream of PKA using an adenylate cyclase inhibitor. This also abolished the effects of ACPT-1. It seems likely then that facilitation of glutamate release induced by activation of presynaptic mGluR4a is dependent on positive coupling to adenylate cyclase and subsequent activation of PKA.

Much of the previous literature concerning presynaptic mGluRs in mammalian CNS has suggested that group II and group III receptors are negatively coupled to adenylate cyclase, and that their activation leads to decreased formation of cAMP and a subsequent reduction in transmitter release. However, evidence does exist for positive coupling of mGluRs to adenylate cyclase (Cartmell et al. 1994; Gereau and Conn 1994; Musgrave et al. 1994; Schoepp and Johnson 1993; Sortino et al. 1996; Winder and Conn 1995). Sciaencalepore et al. (1995) have described an increase in spontaneous GABA release via a mGluR (probably group I) that was also positively coupled to adenylate cyclase in the hippocampus of immature rats. Of particular relevance to our current observations, Zhang et al. (1999) recently reported that activation of a mGluR (analogous to group II) increased glutamate release at the Drosophila neuromuscular junction, an action that occurred via positive coupling to adenylate cyclase and activation of PKA. Our evidence supports a similar effect of the mGluR4a receptor in layer V of the EC. We could not directly assess the effects of PKA and AC inhibition on baseline sEPSC frequency in our experiments, because of the requirement for prolonged incubation in the inhibitors prior to recording. However, at ~2 Hz, mean sEPSC frequency in these experiments was indistinguishable from that under control conditions in untreated slices. Hence our data indicate (albeit indirectly) that baseline sEPSC frequency is not greatly affected by PKA or AC inhibition, and it seems unlikely that the PKA system has a tonic effect on glutamate release in this area. This is consistent with our previous observation that the group III mGluR antagonist, CPPG, had no effect on sEPSC frequency (Evans et al. 2000).

Previous reports from cultured and native hippocampal cells (Finch and Jackson 1990; Hori et al. 1999; Malenka et al. 1986b, 1987; Parfitt and Madison 1993) have shown that activation of PKC with phorbol esters can result in a robust enhancement of transmitter release. We have also demonstrated that PKC can modulate transmitter release in layer V of the EC, but, surprisingly, we did not find a marked facilitation of release in response to PKC activation. Instead, we found that application of the phorbol ester, PMA, did not significantly affect sEPSC frequency, and indeed reduced it at a higher concentration. The demonstration that activation of PKC can influence release does not necessarily show that PKC is the signal used by any given physiological pathway. However, our subsequent studies showed that inhibition of PKC with GF 109203X resulted in a facilitation of glutamate release. This facilitation could be blocked by the specific group III mGluR antagonist CPPG, and by the PKA antagonist H-89. Furthermore, PKC activation prevented the facilitatory effects of mGluR4a activation with APCT-1. Although we could not occlude the effect of forskolin by prior application of GF 109203X, we conclude that the increase in sEPSC frequency observed during blockade of PKC is mediated by the mGluR4a/PKA pathway. These experiments indicate that PKC may be tonically activated in glutamatergic terminals in the
EC, having an overall dampening effect on spontaneous excitatory synaptic transmission.

Hence it seems likely that mGluR4a can increase spontaneous glutamate release by activation of PKA, but this effect is not operating tonically. In contrast, PKC appears to be tonically holding spontaneous transmitter release in check. It is plausible that the lack of tonic activity at mGluR4a receptors may therefore be related to inhibition of mGluR4a or PKA by PKC. If PKC exerts a tonic inhibitory effect on the PKA system, then facilitation of release may only occur after significant activation of PKA via mGluR4a, or on release of either element from inhibition exerted by the PKC system. In fact, there is evidence that activation of PKC inhibits a variety of intracellular pathways, including those involving adenylate cyclase (Jakobs et al. 1985; Kassis et al. 1985; Katada et al. 1985; Mukhopadhyay and Schumacher 1985; Rebois and Patel 1985; Sibley et al. 1984). It is noteworthy that activation of PKC has been shown to greatly reduce the presynaptic inhibitory effects of group II mGluRs at cortico-striatal glutamate synapses (Swartz et al. 1993; Tyler and Lovinger 1995). More recently, Macek et al. (1999) showed that group II and group III mGluR-mediated inhibition of glutamate release is mediated by negative coupling to adenylate cyclase at hippocampal synapses. Activation of PKC suppressed this inhibitory mGluR effect, probably due to uncoupling of the receptors from G-proteins.

Taken together, our results imply that the activity of mGluR4a is regulated by the level of PKC activity within the terminal. It is possible then, that PKC acts as a thresholding device, the activity of which may determine the overall effect of mGluR4a activity and hence the rate of spontaneous glutamate release. At the very least, PKC represents a potential nexus at which cross talk between mGluR4a and other receptor-effector systems may occur.

It remains to be determined at which point(s) between autoreceptor activation and vesicle exocytosis the protein kinases act to modulate synaptic transmission. As discussed above, PKC has recently been reported to inhibit presynaptic group III mGluRs in hippocampus (Macek et al. 1999). This is consistent with our observations, and with the hypothesis that blockade of PKC releases the receptor from tonic inhibition. However, it is equally plausible that PKA and PKC act at other elements of the exocytotic machinery. They may, for example, phosphorylate synaptic proteins (e.g., α-SNAP, SNAP-25, both of which contain consensus sequences for phosphorylation by both PKA and PKC), thereby altering their calcium sensitivity. Alternatively, they may phosphorylate presynaptic Ca<sup>2+</sup> channels (Gubitz et al. 1996), or proteins that regulate release, such as synapsin. In support of the latter hypothesis, there is evidence that synapsins are prominent substrates for PKA (Hosaka et al. 1999), and that phosphorylation results in a facilitation of release (Greengard et al. 1993; Jovanovic et al. 2000). Similarly, experiments on cerebellar neurons indicate that PKA-dependent synaptic facilitation operates via direct modulation of the release machinery (Chavis et al. 1998; Chen and Regehr 1997; Trudeau et al. 1996) and not on factors such as presynaptic Ca<sup>2+</sup> entry. Further experiments aimed at elaboration of the mechanism of this presynaptic mGluR action are under way in our laboratory.

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