

# Priming of Excitatory Synapses by $\alpha_1$ Adrenoceptor-Mediated Inhibition of Group III Metabotropic Glutamate Receptors

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Adaptive responses mediated by the hypothalamus require sustained activation until homeostasis is achieved. Increases in excitatory drive to the magnocellular neuroendocrine cells that mediate these responses, however, result in the activation of a presynaptic metabotropic glutamate receptor (mGluR) that curtails synaptic excitability. Recent evidence that group III mGluRs can be inhibited by protein kinase C prompted us to test the hypothesis that activation of PKC by noradrenaline (NA) inhibits group III mGluRs and increases excitatory synaptic input to these cells. To examine the effects of NA on miniature EPSCs (mEPSCs), we obtained whole-cell recordings from magnocellular vasopressin and oxytocin neurons in the paraventricular nucleus of the hypothalamus. All of the neurons tested in the current study displayed an  $\alpha_1$  adrenoceptor-mediated increase in mEPSC frequency in response to NA (1–200  $\mu\text{M}$ ). The excitatory effects of NA were mimicked by the phorbol ester PMA and blocked by the PKC inhibitor calphostin C. The activation of PKC inhibits the efficacy of group III mGluRs, resulting in an increase in mEPSC frequency in response to a subsequent exposure to NA. By removing feedback inhibition, this mechanism effectively primes the synapses such that subsequent activation is more efficacious. The novel form of synaptic rescaling afforded by this cross-talk between distinct metabotropic receptors provides a means by which ascending catecholamine inputs can facilitate the control of homeostasis by hypothalamic networks.

**Key words:** magnocellular; hypothalamus; presynaptic inhibition; G-protein-coupled receptors; noradrenaline; paraventricular nucleus

## Introduction

In the CNS, the release of neurotransmitter from the nerve terminal is regulated by a number of inhibitory feedback mechanisms (Zucker and Regehr, 2002). These mechanisms include, but are not limited to, the activation of high-affinity presynaptic receptors by neurotransmitters such as acetylcholine (MacDermott et al., 1999), adenosine (Oliet and Poulain, 1999; Dunwiddie and Masino, 2001), or GABA (Mouginot et al., 1998). At the majority of excitatory synapses, however, this feedback is initiated by the binding of glutamate to presynaptic, G-protein-coupled autoreceptors (for review, see Schoepp, 2001). Activation of these metabotropic glutamate receptors (mGluRs) results in a decrease in the release of neurotransmitter (Baskys and Malenka, 1991; Schoppa and Westbrook, 1997), thereby providing a local, short-term mechanism through which synaptic strength (Anwyl, 1991; Scanziani et al., 1997; Oliet et al., 2001) and hyperexcitability (Sansig et al., 2001) may be regulated.

In certain physiological situations, however, neuronal output must be sustained for longer durations. Under these conditions, a process that limits the continuous release of glutamate may be

unfavorable. In particular, adaptive responses mediated by the hypothalamus generally require the sustained activation of neuronal populations until homeostasis is achieved. In the supraoptic nucleus (SON) and paraventricular nucleus (PVN), the magnocellular neuroendocrine cells exhibit prolonged discharges in response to specific physiological perturbations (Wakerley et al., 1978). This increase in neuronal activity may be achieved by changes in intrinsic conductances (Bourque and Renaud, 1984; Legendre and Poulain, 1992; Hatton and Li, 1998; Shibuya et al., 2000) or, alternatively, by a persistent increase in excitatory synaptic drive (Nissen et al., 1995; Moos et al., 1997; Jourdain et al., 1998; Shibuya et al., 2000) to these neurons. Because this glutamatergic input to magnocellular neuroendocrine cells is regulated by presynaptic mGluRs (Schrader and Tasker, 1997; Oliet et al., 2001), this raises the possibility that targeting mGluR autoreceptor activity may be an attractive means for potentiating this input and ultimately augmenting the output of these neurons.

Recent evidence demonstrates that an increase in protein kinase C activity functionally inhibits mGluRs (Macek et al., 1998, 1999; Nakajima et al., 1999). Thus, substrates that activate signaling pathways linked to PKC may exert long-lasting changes in synaptic efficacy by decreasing mGluR activity. In the hypothalamus, the physiological trigger for this override of mGluR feedback may be the activation of presynaptic, G-protein-coupled  $\alpha_1$  adrenoceptors by noradrenaline (NA), which is released from ascending afferents in response to a number of physiological challenges (Crowley et al., 1987; Leibowitz et al., 1990). Because

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$\alpha_1$  adrenoceptors are positively coupled to PKC, we hypothesize that the functional inactivation of mGluRs by NA primes excitatory glutamatergic synapses terminating on magnocellular neuroendocrine cells.

Using whole-cell, voltage-clamp recordings from magnocellular neurons in the PVN of the hypothalamus, we demonstrate that  $\alpha_1$  adrenoceptor activation increases glutamate release and inactivates presynaptic mGluRs. This PKC-mediated inhibition of negative feedback is long lasting, as evidenced by an amplification of transmitter release in response to subsequent application of NA. The rescaling of synaptic input that results from cross-talk between distinct metabotropic receptors provides a means by which ascending catecholamine inputs can facilitate the control of homeostasis by hypothalamic networks.

## Materials and Methods

Hypothalamic slices containing the PVN were prepared from young adult, postnatal day (P) 21–27, male Sprague Dawley rats. Animals were anesthetized with sodium pentobarbital (3 mg/kg) and decapitated, and the brains were rapidly removed into ice-cold high-sucrose slicing solution saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> and allowed to cool for ~3 min. The brain was then blocked and mounted on a vibrating slicer (Leica, Nussloch, Germany) and submerged in ice-cold slicing solution containing the following (in mM): 87 NaCl, 2.5 KCl, 25 NaHCO<sub>3</sub>, 0.5 CaCl<sub>2</sub>, 7 MgCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 25 glucose, 75 sucrose, 3 pyruvic acid, and 1 ascorbic acid, saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The brain was cut in the coronal plane, and hemisected slices of 300  $\mu$ m thickness containing the hypothalamus were incubated at 32.5°C in a submerged chamber of artificial CSF (ACSF) for a minimum of 60 min before recording. ACSF contained the following (in mM): 126 NaCl, 2.5 KCl, 26 NaHCO<sub>3</sub>, 2 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 10 glucose, and 1 ascorbic acid, saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Whole-cell recordings were obtained from magnocellular neurons visually identified using an upright microscope (AxioskopII FS Plus; Zeiss, Oberkochen, Germany) fitted with infrared differential interference contrast. All recordings were obtained at 32.5°C using borosilicate glass microelectrodes (tip resistance, 3–7 M $\Omega$ ). The intracellular solution contained the following (in mM): 123 potassium gluconate, 2 MgCl<sub>2</sub>, 8 NaCl, 1 potassium EGTA, 4 potassium ATP, and 0.3 sodium GTP, buffered with 16 mM KHCO<sub>3</sub>. The internal solution was filtered before use.

With the exception of the initial confirmation of cell type based on the electrical fingerprint in bridge mode, all experiments were performed in voltage-clamp mode, and recordings were accepted when access resistance changes were limited to <15%. All experiments were performed on PVN magnocellular neurons first identified visually and confirmed by their prominent delay to first spike in response to depolarizing current steps from hyperpolarized potentials (Tasker and Dudek, 1991). The perfusate always contained picrotoxin (100  $\mu$ M) to block inhibitory GABA<sub>A</sub> synaptic currents and tetrodotoxin (1  $\mu$ M) to block voltage-gated Na<sup>+</sup> channel and inhibit action potential-driven release of neurotransmitter.

Signals were amplified with the Multiclamp 700A amplifier (Axon Instruments, Foster City, CA), low-pass filtered at 1 kHz, and digitized at 5–10 kHz using the Digidata 1322 (Axon Instruments). Data were collected (pClamp; Axon Instruments) and stored on computer for off-line analysis using software designed to detect miniature synaptic events using a variable threshold (MiniAnalysis; Synaptosoft, Decatur, GA). Experimental values are presented as means  $\pm$  SEM, and statistical analyses were performed using Student's *t* test when comparing two groups and ANOVA with a *post hoc* Newman–Keuls test for comparisons across multiple groups. *p* < 0.05 was accepted as statistically significant (\**p* < 0.05; \*\**p* < 0.01).

Reagents were obtained from the following sources. TTX, picrotoxin, calphostin C, PMA, 1-[3,4-dihydroxyphenyl]-2-aminoethanol (NA/arterenol), and 1-[4-amino-6,7-dimethoxy-2-quinazolinyl]-4-[2-furanyl-carbonyl]-piperazine (prazosin), were purchased from Sigma (St. Louis, MO). TTX was also purchased from Alomone Labs (Jerusalem, Israel).

L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) and (S)-2-amino-2-methyl-4-phosphonobutanoic acid (MAP4) were purchased from Tocris Cookson (Ballwin, MO). Picrotoxin and calphostin C were dissolved in dimethylsulfoxide in which the final bath concentration of DMSO was <0.1%. Prazosin was dissolved in methanol, in which the final bath concentration of methanol was <0.05%. All other reagents were dissolved in ACSF.

## Results

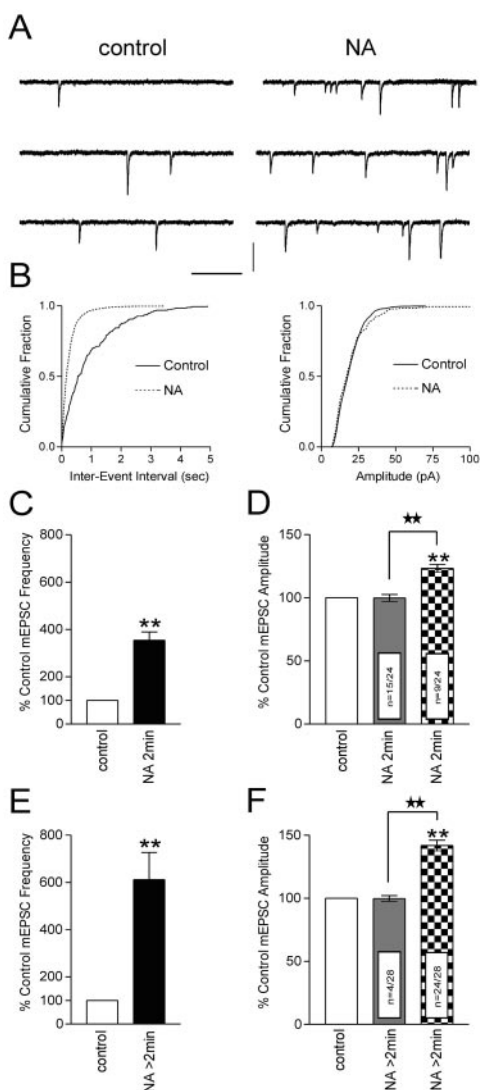
In contrast to neurons in most cortical regions in which the spontaneous, action potential-independent release of neurotransmitter is relatively infrequent (Staley, 1999), the magnocellular neuroendocrine cells of the hypothalamus are subjected to relatively high rates of quantal input. In the cells tested in the current study, the rate of stochastic release under basal conditions ranged from 0.46 to 8 Hz (mean, 2.74  $\pm$  0.35 Hz). This high frequency of events combined with the demonstration that in magnocellular neurons, increases in mEPSC frequency lead to increases in firing (Kombian et al., 2000) suggests that quantal glutamatergic release conveys important signaling information to these neurons.

NA exerts predominantly excitatory effects on magnocellular neurons (Day et al., 1984; Armstrong et al., 1986; Randle et al., 1986b). These are partially attributable to a direct  $\alpha_1$ -mediated depolarization of the postsynaptic membrane (Randle et al., 1986a) and an increase in TTX-sensitive glutamate release (Daftary et al., 1998). The effects of NA on quantal release at the presynaptic nerve terminal, however, have been less well defined. To clarify a role for NA at afferent excitatory terminals synapsing on magnocellular neurons, we examined the effects of this compound on TTX-insensitive mEPSCs.

### NA increases the frequency and amplitude of mEPSCs

In response to a brief application of NA (2 min, 100  $\mu$ M), all of the neurons tested exhibited an increase in mEPSC frequency (354.2  $\pm$  34.9% of control; *n* = 24; *p* < 0.01) (Fig. 1A–C). Although in the majority of cells tested (63%, *n* = 15 of 24) NA had no effect on mEPSC amplitude (99.8  $\pm$  2.8% of control; *p* > 0.05) (Fig. 1B,D), an increase in amplitude was observed in the remaining cells (123.3  $\pm$  3.0% of control; *p* < 0.01) (Fig. 1D). We also noted a mean inward current of 22.1  $\pm$  2.2 pA in cells tested at 100  $\mu$ M (*p* < 0.05; data not shown), consistent with a direct postsynaptic effect of NA on magnocellular neurons (Randle et al., 1986a; Daftary et al., 1998). We next tested the effects of longer NA applications on mEPSC frequency and amplitude. In response to NA applications that ranged from 4 to 10 min, the mEPSC frequency increased to 612.2  $\pm$  114.2% of control (*p* < 0.01; *n* = 28) (Fig. 1E). Interestingly, in these longer applications, the majority of cells (86%; *n* = 24 of 28) exhibited an increase in mEPSC amplitude (141.8  $\pm$  4.2% of control; *p* < 0.01) (Fig. 1F). The remaining cells (14%; *n* = 4 of 28) displayed no change in mEPSC amplitude (99.7  $\pm$  2.3% of control; *p* > 0.05) (Fig. 1F). This discrepancy in response profiles for short versus long applications of NA suggests a time-dependent component to the excitation induced by NA.

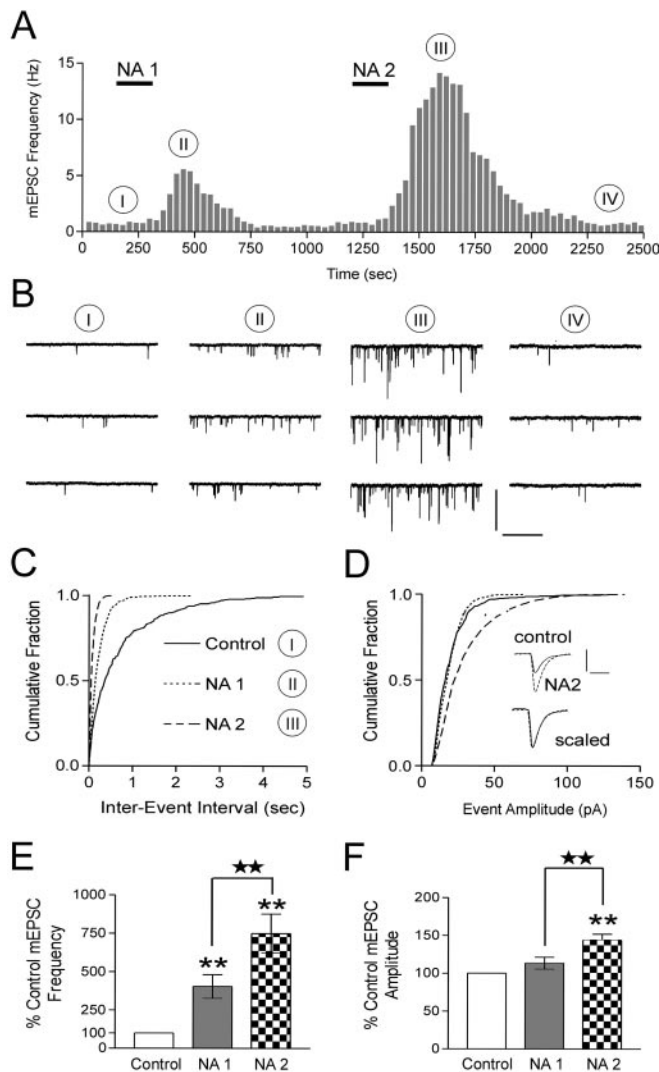
The effects of NA were also dose dependent. Cells tested with brief applications of 1, 10, and 200  $\mu$ M NA exhibited increases in mEPSC frequency of 104.6  $\pm$  8.6% (*n* = 2), 188.4  $\pm$  14.9% (*n* = 6), and 877.6  $\pm$  37.8% (*n* = 2), respectively (data not shown). No inward current was observed at 1  $\mu$ M NA, whereas 10  $\mu$ M NA elicited significantly less inward current compared with 100  $\mu$ M treatments (9.7  $\pm$  2.4 pA; *p* < 0.05; *n* = 3; data not shown). Because of the robust increase in mEPSC frequency, we were unable to accurately assess changes in holding current for 200  $\mu$ M NA.



**Figure 1.** NA increases frequency and amplitude of mEPSCs. *A*, Representative voltage-clamp traces from a cell tested with a 2 min application of NA (100  $\mu$ M). *B*, In the cumulative fraction plots, NA decreases the inter-event interval (left,  $p < 0.01$ ) with no change in mEPSC amplitude (right,  $p > 0.05$ ). *C–F*, Summary bar graphs of NA data. *C*, A 2 min application of NA increases mEPSC frequency to  $354.2 \pm 34.9\%$  of control ( $p < 0.01$ ;  $n = 24$ ). *D*, In 63% of these cells ( $n = 15$  of 24), there is no change in mEPSC amplitude ( $99.8 \pm 2.8\%$  of control;  $p > 0.05$ ). The remaining 37% of cells tested ( $n = 9$  of 24) exhibit an increase in mEPSC amplitude ( $123.3 \pm 3.0\%$  of control;  $p < 0.01$ ). *E*, When the NA application exceeds 2 min, the mEPSC frequency increases to  $612.2 \pm 114.2\%$  of control ( $p < 0.01$ ;  $n = 28$ ). *F*, In these cells, 86% ( $n = 24$  of 28) exhibited an increase in mEPSC amplitude ( $141.8 \pm 4.2\%$  of control;  $p < 0.01$ ). No change in mEPSC amplitude ( $99.7 \pm 2.3\%$  of control;  $p > 0.05$ ) was observed in the remaining 14% ( $n = 4$  of 28). Calibration (in *A*): 15 pA, 250 msec. Stars indicate comparing treatment with treatment. Asterisks indicate comparing treatment with control.

### NA primes glutamatergic synapses

Sustained increases in neuronal activity and hormonal output are reliably observed in magnocellular neuroendocrine cells in response to acute physiological stressors such as dehydration or hemorrhage. Although a single hemorrhagic stimulus elicits a sustained increase in hormone release from these cells, repeated hemorrhages result in an amplification of hormone release (Lilly et al., 1989). We examined whether repeated applications of NA would mimic these observations and provide an explanation at the synaptic level for the amplification of hormone release. We conducted experiments that used brief (2 min) repetitive applications of NA. The initial application of NA elicited an increase



**Figure 2.** NA primes excitatory synapses. *A*, Two 2 min applications of NA (100  $\mu$ M) separated by 15 min demonstrate an enhancement of the mEPSC frequency from NA1 to NA2. *B*, Representative voltage-clamp traces from the same neuron as in *A*. The roman numerals in *B* correspond to the same time point in *A*. *C*, Cumulative fraction of mEPSC inter-event intervals before and during each NA application in this neuron. NA2 elicits an additional decrease in mEPSC inter-event interval ( $p < 0.01$ ). *D*, Cumulative fraction of the amplitude of mEPSCs before and during each NA application. Here, only NA2 results in a significant increase in mEPSC amplitude ( $p < 0.05$ ). Inset traces are averages of 40 spontaneous events in control and NA2. Control and NA2 traces are overlaid below to demonstrate that no change in the kinetics of the mEPSCs is observed. *E*, Summary bar graph showing the increase in mEPSC frequency after each successive NA application (NA1,  $403.8 \pm 77.2\%$  of control;  $p < 0.01$ ;  $n = 6$ ). The mEPSC frequencies from NA1 and NA2 are significantly different ( $p < 0.05$ ). *F*, Summary bar graph of increases in mEPSC amplitude (NA1,  $113.4 \pm 7.8\%$  of control;  $p > 0.05$ ; NA2,  $143.9 \pm 7.8\%$  of control;  $p < 0.01$ ,  $n = 6$ ). The mEPSC amplitudes from NA1 and NA2 are significantly different ( $p < 0.05$ ). Calibration: *B*, 50 pA, 1 sec; *D*, 20 pA, 5 msec. Stars indicate comparing treatment with treatment. Asterisks indicate comparing treatment with control.

(Fig. 2*A–C*) in the frequency of mEPSCs ( $403.8 \pm 77.2\%$  of control;  $n = 6$ ;  $p < 0.01$ ) (Fig. 2*E*). After the recovery of mEPSC frequency to control levels, the synapses were rechallenge for 2 min with the same dose of NA. This application (15 min after the initial test) resulted in an additional amplification (Fig. 2*A–C*) in the mEPSC frequency ( $749.8 \pm 126.4\%$  of control;  $n = 6$ ;  $p < 0.01$ ) (Fig. 2*E*). We also observed an increase in mEPSC amplitude in response to the second application of NA only (Fig. 2*B,D*). We did not observe any changes in the kinetics of the

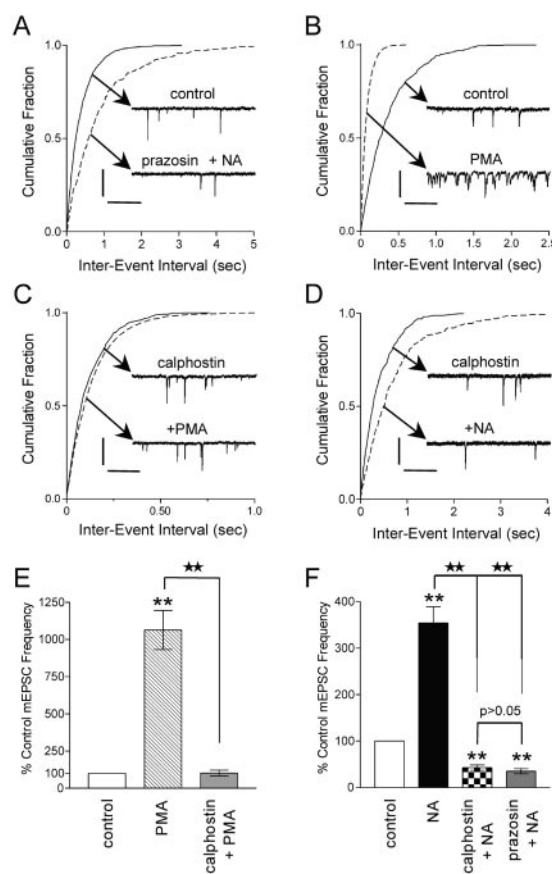
mEPSCs after application of NA (Fig. 2D, inset scaled traces). The mEPSC amplitude increased from  $113.4 \pm 7.8\%$  ( $p > 0.05$ ) of control to  $143.9 \pm 7.8\%$  ( $p < 0.05$ ) (Fig. 2F) of control for NA1 and NA2, respectively. Additional applications of NA failed to increase the frequency of mEPSCs further ( $n = 3$ ; data not shown). Our analysis failed to reveal any consistent changes in amplitude for subsequent applications. These results indicate that NA primes excitatory synapses for subsequent exposure to the agonist.

### Increase in mEPSC frequency is $\alpha_1$ adrenoceptor mediated and PKC dependent

The excitatory effects of NA on magnocellular neurons involve the activation of the  $\alpha_1$  adrenoceptor (Day et al., 1984; Armstrong et al., 1986; Randle et al., 1986b). These effects have been characterized as a direct  $\alpha_1$ -mediated depolarization of the postsynaptic membrane and an  $\alpha_1$ -mediated increase in TTX-sensitive glutamate release (Randle et al., 1986a; Daftary et al., 1998). On the basis of these findings, we tested whether activation of the  $\alpha_1$  adrenoceptor was necessary for the NA-induced increase in mEPSC frequency. The  $\alpha_1$  adrenoceptor antagonist prazosin (10  $\mu\text{M}$ ) completely abolished the excitatory effects of NA, uncovering an inhibitory action (mEPSC frequency decreased to  $35.2 \pm 5.2\%$  of control values;  $p < 0.01$ ;  $n = 12$ ) (Fig. 3A,F). These data are consistent with previous results demonstrating an  $\alpha_1$ -mediated increase in spontaneous IPSCs in PVN neurons that masks a weaker inhibition of GABA release via  $\alpha_2$  adrenoceptor (Han et al., 2002). Our results suggest a similar role for NA at excitatory synapses. These findings demonstrate that NA acts predominantly via presynaptic  $\alpha_1$  adrenoceptors to increase glutamate release from afferent nerve terminals. This increase in mEPSC frequency likely acts in concert with the previously defined actions of this catecholamine in sustaining the elevated activity of these cells.

The  $\alpha_1$  adrenoceptor activates phospholipase C through the Gq family of G-proteins, resulting in phosphatidylinositol metabolism, the release of stored  $\text{Ca}^{2+}$ , and activation of PKC (for review, see Zhong and Minneman, 1999). PKC activation mobilizes the reserve pool of vesicles (Gillis et al., 1996; Stevens and Sullivan, 1998), increases the sensitivity of the release process for  $\text{Ca}^{2+}$  (Brager et al., 2002), and facilitates the fusion of vesicles (Scepek et al., 1998; Yawo, 1999). These mechanisms may act individually or in concert to increase neurotransmitter release. Phorbol esters, such as PMA, activate PKC by binding to the C1 domain in an ATP-dependent manner (Liu and Heckman, 1998). The nonphysiological activation of PKC by lipid-soluble phorbol esters has been used widely for discerning the effects of the kinase in a number of neuronal and neuroendocrine preparations (Hilfiker and Augustine, 1999). To test the hypothesis that phorbol esters mimic the effects of NA, we examined mEPSC frequency in response to PMA (1  $\mu\text{M}$ ) (Fig. 3B). mEPSC frequency dramatically increased ( $1063.5 \pm 131.4\%$  of control;  $n = 5$ ;  $p < 0.01$ ) (Fig. 3E) in response to a 10 min PMA application. We observed no change in mEPSC amplitude (data not shown). To confirm that this increase in release was a result of the activation of PKC, the slice was incubated for 30 min with the broad-spectrum PKC inhibitor calphostin C (100 nM) (Hasuo et al., 2002), and the PMA application was repeated (Fig. 3C). In the presence of calphostin C, PMA treatment had no effect on mEPSC frequency ( $102.1 \pm 19.5\%$  of control;  $n = 5$ ;  $p > 0.05$ ) (Fig. 3E). These data suggest that PMA acts presynaptically to activate PKC and increase the release of glutamate.

Experiments were conducted to test whether PKC activation

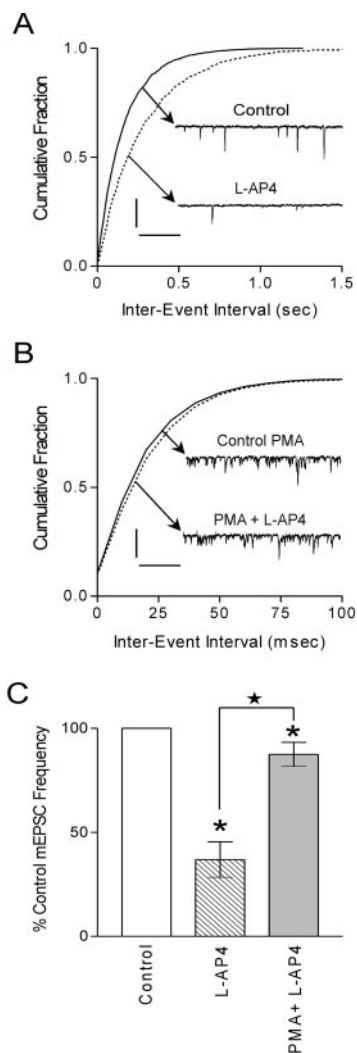


**Figure 3.** Increase in mEPSC frequency is  $\alpha_1$  adrenoceptor mediated and PKC dependent. *A*, Cumulative fraction of mEPSC inter-event intervals before and during prazosin (10  $\mu\text{M}$ ) plus NA (100  $\mu\text{M}$ ) application. Inset are voltage-clamp traces depicting a decrease in the mEPSC frequency in response to NA when the  $\alpha_1$  adrenoceptor is blocked ( $p < 0.01$ ). *B*, Cumulative fraction of mEPSC inter-event intervals before and during PMA (1  $\mu\text{M}$ ) application. Inset are voltage-clamp traces depicting an increase in the mEPSC frequency in response to PMA ( $p < 0.01$ ). *C*, Cumulative fraction of mEPSC inter-event intervals in response to PMA (1  $\mu\text{M}$ ) in the presence of calphostin C (100 nM). Inset are voltage-clamp traces depicting that no change in mEPSC frequency in response to PMA in the presence of calphostin C ( $p > 0.05$ ) is observed. *D*, Cumulative fraction of mEPSC inter-event interval in response to NA (100  $\mu\text{M}$ ), in the presence of calphostin C (100 nM). Inset are voltage-clamp traces depicting a decrease in mEPSC frequency in response to NA in calphostin C ( $p < 0.05$ ). *E*, Summary bar graph showing that PMA alone increases mEPSC frequency ( $1063.5 \pm 131.4\%$  of control;  $p < 0.01$ ;  $n = 5$ ) but fails to increase mEPSC frequency in the presence of calphostin C ( $102.1 \pm 19.5\%$  of control;  $n = 5$ ;  $p > 0.05$ ). *F*, Summary bar graph showing that NA decreases the frequency of mEPSCs in the presence of calphostin C ( $43.0 \pm 5.7\%$  of control;  $p < 0.01$ ;  $n = 5$ ) or prazosin ( $35.2 \pm 5.2\%$  of control;  $p < 0.01$ ;  $n = 12$ ). Calibration: *A*, 20 pA, 250 msec; *B*, 25 pA, 100 msec; *C*, 20 pA, 250 msec; *D*, 15 pA, 250 msec. Stars indicate comparing treatment with treatment. Asterisks indicate comparing treatment with control.

was necessary for the NA-mediated increase in glutamate release. Slices were preincubated for 30 min with calphostin C (100 nM), and NA was applied at a dose (100  $\mu\text{M}$ ) that normally elicits a robust increase in mEPSC frequency (Fig. 3D). Calphostin C completely abolished the NA-induced increase in mEPSC frequency and unmasked an inhibitory effect (mEPSC frequency decreased to  $43.0 \pm 5.7\%$  of control;  $p < 0.01$ ;  $n = 5$ ) (Fig. 3F).

### Phorbol ester and NA-induced PKC activation can attenuate inhibitory mGluR activity

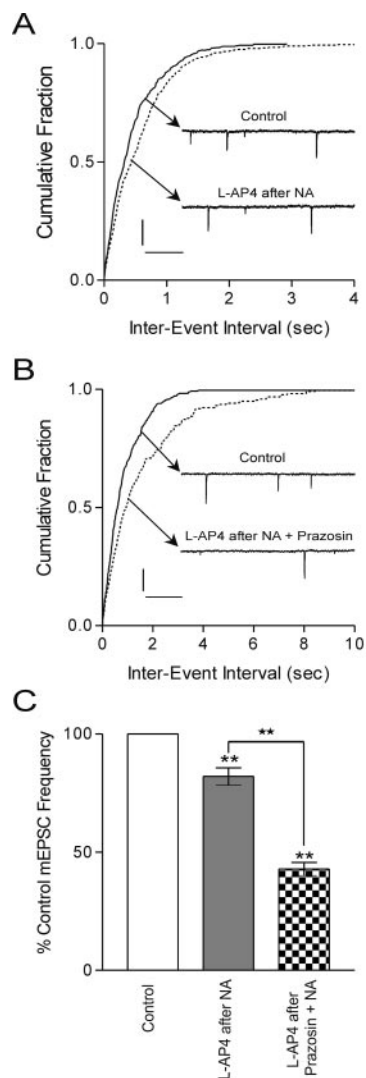
Membrane-binding assays have established a robust distribution of mGluRs in the hypothalamus (Meeker et al., 1994). In particular, electrophysiological evidence demonstrates an important role for group III mGluRs in regulating glutamate release at mag-



**Figure 4.** PKC activation attenuates mGluR-mediated inhibition. *A*, Cumulative fraction of mEPSC inter-event interval before and during L-AP4 (25  $\mu$ M) application. Inset are voltage-clamp traces depicting a decrease in the mEPSC frequency in response to L-AP4 ( $p < 0.01$ ). *B*, Cumulative fraction of mEPSC inter-event interval in response to L-AP4 (25  $\mu$ M) in the presence of PMA (1  $\mu$ M). Inset are voltage-clamp traces depicting a small decrease in mEPSC frequency in response to L-AP4 in PMA ( $p > 0.05$ ). *C*, Summary bar graph demonstrates that L-AP4 decreases mEPSC frequency ( $36.9 \pm 8.5\%$  of control;  $p < 0.05$ ;  $n = 3$ ). In the presence of PMA, L-AP4 exhibits a small decrease in mEPSC frequency ( $87.5 \pm 5.8\%$  of control;  $p < 0.05$ ;  $n = 5$ ). The decrease in mEPSC frequency in L-AP4 is significantly different from that observed in PMA plus L-AP4 ( $p < 0.05$ ). Calibration: *A*, 20 pA, 250 msec; *B*, 25 pA, 100 msec. Stars indicate comparing treatment with treatment. Asterisks indicate comparing treatment with control.

nocellular synapses (Schrader and Tasker, 1997; Oliet et al., 2001). We confirmed this finding with the demonstration that bath application of the group III mGluR agonist L-AP4 (25  $\mu$ M) decreased the frequency of mEPSCs in magnocellular neurons ( $36.9 \pm 8.5\%$ ;  $n = 3$ ;  $p < 0.05$ ) (Fig. 4*A,C*). No apparent postsynaptic effects of the agonist were observed (data not shown).

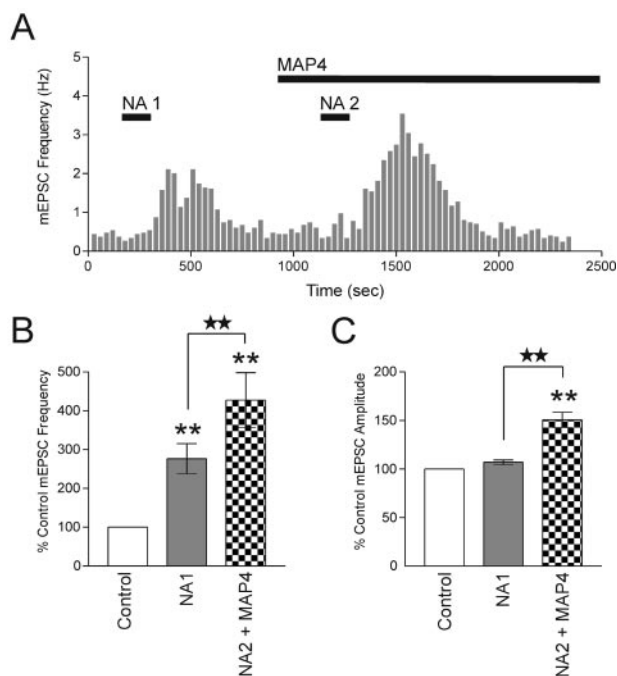
mGluRs themselves are also subject to regulation by intracellular messengers. Specifically, some studies propose the inhibition of group III mGluRs by PKC (Macek et al., 1999; Sorensen et al., 2002). To test the hypothesis that mGluR function would be compromised after the activation of PKC, we applied PMA (1  $\mu$ M) and then L-AP4 (25  $\mu$ M) 15 min later. As demonstrated above, PMA application elicited a robust increase in mEPSC frequency. In the presence of PMA, the effect of L-AP4 on mEPSC frequency was blunted ( $87.5 \pm 5.8\%$  of control;  $n = 5$ ;  $p < 0.05$ )



**Figure 5.**  $\alpha_1$  adrenoceptor activation attenuates mGluR-mediated inhibition. *A*, Cumulative fraction of mEPSC inter-event interval before and during L-AP4 (25  $\mu$ M) after application of NA (100  $\mu$ M). Inset are voltage-clamp traces depicting a small decrease in mEPSC frequency in response to L-AP4 after NA ( $p < 0.05$ ). *B*, Cumulative fraction of mEPSC inter-event interval before and during L-AP4 (25  $\mu$ M) after application of prazosin (10  $\mu$ M) plus NA (100  $\mu$ M). Inset are voltage-clamp traces depicting a clear decrease in mEPSC frequency in response to L-AP4 after NA when the  $\alpha_1$  adrenoceptor is blocked ( $p < 0.01$ ). *C*, Summary bar graph showing a decrease in the frequency of mEPSCs with L-AP4 after NA ( $82.1 \pm 3.7\%$  of control;  $p < 0.01$ ;  $n = 5$ ) and with L-AP4 after prazosin plus NA ( $42.9 \pm 2.8\%$  of control;  $p < 0.01$ ;  $n = 7$ ). The decrease in mEPSC frequency after L-AP4 after NA is significantly different from that observed in L-AP4 after prazosin plus NA ( $p < 0.01$ ). Calibration: *A*, 20 pA, 250 msec; *B*, 25 pA, 200 msec. Stars indicate comparing treatment with treatment. Asterisks indicate comparing treatment with control.

(Fig. 4*B,C*). This response was significantly different from L-AP4 alone ( $p < 0.05$ ). These data suggest that PKC activation attenuates the decrease in glutamate release normally observed in response to activation of group III mGluRs on the presynaptic terminal.

We next examined whether activation of PKC by NA would also attenuate mGluR function. After a return to baseline of the NA-induced increase in mEPSC frequency, L-AP4 was applied at a dose that had previously decreased mEPSC frequency (25  $\mu$ M) (Fig. 5*A*). Under these conditions, the effect of L-AP4 on mEPSC frequency was significantly attenuated (L-AP4 after NA;  $82.1 \pm 3.7\%$  of control;  $p < 0.01$ ;  $n = 5$ ) (Fig. 5*C*) compared with L-AP4

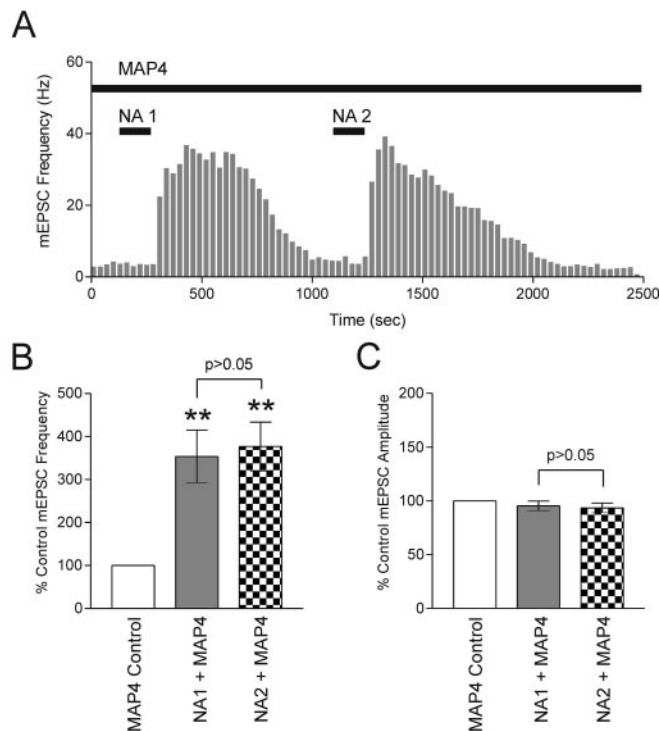


**Figure 6.** Blockade of group III mGluRs after NA1 does not affect priming. *A*, The priming effect of NA is still observed when NA2 is applied in the presence of MAP4 (250  $\mu$ M). NA2 (2 min, 100  $\mu$ M) shows a greater increase in mEPSC frequency than NA1 (2 min, 100  $\mu$ M). *B*, Summary bar graph of increases in mEPSC frequency (NA1, 276.3  $\pm$  38.9% of control;  $p$  < 0.01; NA2, 427.0  $\pm$  70.5% of control;  $p$  < 0.01;  $n$  = 7). The mEPSC frequencies from NA1 and NA2 are significantly different ( $p$  < 0.01). *C*, Summary bar graph showing effects of NA on mEPSC amplitude (NA1, 107.0  $\pm$  2.4% of control;  $p$  > 0.05; NA2, 150.6  $\pm$  7.9%;  $p$  < 0.01;  $n$  = 5). The mEPSC amplitudes from NA1 and NA2 are significantly different ( $p$  < 0.01). Stars indicate comparing treatment with treatment. Asterisks indicate comparing treatment with control.

alone ( $p$  < 0.01) (Fig. 4*A,C*, comparison not shown). Finally, we tested whether activation of the  $\alpha_1$  adrenoceptor by NA was necessary for the inhibition of group III mGluRs. After the application of NA in the presence of prazosin (to block  $\alpha_1$  adrenoceptors), L-AP4 clearly decreased the frequency of mEPSCs (42.9  $\pm$  2.8% of control;  $p$  < 0.01;  $n$  = 7) (Fig. 5*B,C*). This was significantly different from the effects of L-AP4 after NA alone ( $p$  < 0.01) but was not different from L-AP4 alone ( $p$  > 0.05) (Fig. 4*C*, comparison not shown).

### Blockade of group III mGluRs after NA1 does not affect priming

We next tested whether pharmacological inhibition of group III mGluRs during the second NA application would alter the priming response. If synaptic priming occurs because of different activation states of the mGluR (where less glutamate release during NA1 is attributed to a functional mGluR and more glutamate release during NA2 is attributed to mGluR inactivation), then inhibiting the mGluR during NA2 should have little effect on priming. The priming experiment was repeated, but instead, the group III mGluR antagonist MAP4 (250  $\mu$ M) was bath applied after NA1 (Fig. 6*A*). The initial application of NA increased the frequency of mEPSCs to 276.3  $\pm$  38.9% of control ( $p$  < 0.01;  $n$  = 7) (Fig. 6*A,B*). After the recovery of mEPSC frequency to control levels, the synapses were rechallenged with NA in the presence of MAP4. This resulted in an additional amplification in the mEPSC frequency to 427.0  $\pm$  70.5% of control ( $p$  < 0.01;  $n$  = 7) (Fig. 6*A,B*). The responses elicited by NA1 and NA2 are significantly different ( $p$  < 0.01) (Fig. 6*B*). Consistent with the results from

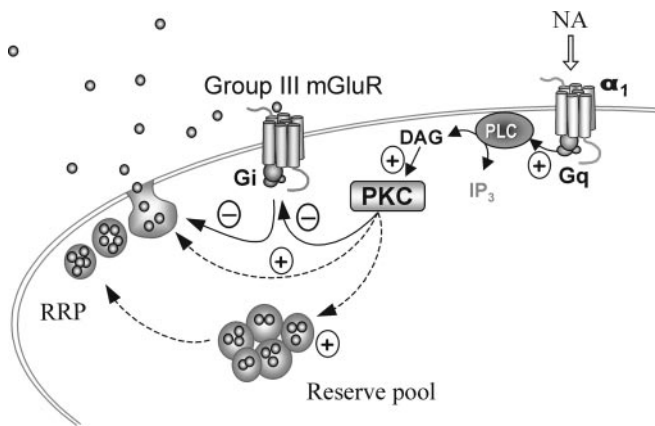


**Figure 7.** Blockade of group III mGluRs abolishes NA-induced priming. *A*, In the presence of MAP4 (250  $\mu$ M), two 2 min applications of NA (100  $\mu$ M) separated by 15 min exhibit similar increases in mEPSC frequency. *B*, Summary bar graph of increases in mEPSC frequency (NA1, 353.5  $\pm$  61.1% of control;  $p$  < 0.01; NA2, 376.5  $\pm$  57.0% of control;  $p$  < 0.01;  $n$  = 4). The mEPSC frequencies from NA1 and NA2 are not significantly different ( $p$  > 0.05). *C*, Summary bar graph showing effects of NA on mEPSC amplitude (NA1, 95.4  $\pm$  4.4% of control;  $p$  > 0.05; NA2, 93.4  $\pm$  4.5%;  $p$  > 0.05;  $n$  = 4). The mEPSC amplitudes from NA1 and NA2 are not significantly different ( $p$  > 0.05). Asterisks indicate comparing treatment with control.

earlier experiments, we observed an increase in mEPSC amplitude in response to the second application of NA (Fig. 6*C*). The mEPSC amplitude increased from 107.0  $\pm$  2.4% ( $p$  > 0.05) of control to 150.6  $\pm$  7.9% ( $p$  < 0.01;  $n$  = 5) (Fig. 6*C*) of control for NA1 and NA2, respectively. These data collectively suggest that mGluRs are inactivated after NA application. Consequently, they are unavailable to curtail the release of glutamate when the synapses are rechallenged with NA.

### Group III mGluRs mediate priming

Finally, we tested the inhibitory contribution of group III mGluRs during the initial response to NA. If synaptic priming is caused by differences in mGluR activity during NA1 and NA2, then inhibiting the mGluR for the duration of NA1 and NA2 application should potentiate release under both conditions, effectively eliminating the observation of "priming." In the presence of MAP4, NA elicited a robust increase in mEPSC frequency (353.5  $\pm$  61.1%;  $n$  = 4;  $p$  < 0.01) (Fig. 7*A,B*) but had no effect on mEPSC amplitude (95.4  $\pm$  4.4%;  $n$  = 4;  $p$  > 0.05) (Fig. 7*C*). We failed to see any more potentiation when NA was applied a second time in the presence of MAP4. The effect on mEPSC frequency (376.5  $\pm$  57%;  $p$  < 0.01) (Fig. 7*A,B*) and amplitude (93.5  $\pm$  4.5%;  $p$  > 0.05) (Fig. 7*C*) by NA2 was not different from the response to the first application ( $p$  > 0.05) (Fig. 7*B,C*). The absence of any change in amplitude may be because of the continuous application of MAP4 for the duration of the experiment. The demonstration that inhibition of presynaptic mGluRs increases the amplitude of spontaneous EPSCs (Bandrowski et al.,



**Figure 8.** Cross-talk between metabotropic receptor signaling pathways. Schematic illustrates the putative interaction between the presynaptic  $\alpha_1$  adrenoceptor and group III mGluRs.  $\alpha_1$ ,  $\alpha_1$  Adrenoceptor; PLC, phospholipase C; IP<sub>3</sub>, inositol triphosphate; DAG, diacylglycerol; PKC, protein kinase C; RRP, readily releasable pool; NA, noradrenaline.

2003) is consistent with this idea. We tested this possibility in a separate set of experiments by applying MAP4 in the presence of TTX. Under these conditions, MAP4 significantly increased the amplitude of mEPSCs ( $121.7 \pm 1.9\%$  of control;  $p < 0.01$ ;  $n = 6$ ; data not shown). In light of this evidence, it is plausible that the priming of both mEPSC frequency and amplitude results from the functional inactivation of presynaptic autoreceptors. Together, these data suggest that activation of  $\alpha_1$  adrenoceptors by NA curtails the efficacy of group III mGluRs via a PKC-dependent mechanism to prime the glutamatergic synapse for subsequent exposure of the agonist (Fig. 8).

## Discussion

Our findings demonstrate that the activation of PKC via  $\alpha_1$  adrenoceptors not only increases the frequency of mEPSCs but also functionally attenuates presynaptic group III mGluRs. Moreover, by removing mGluR-mediated feedback inhibition,  $\alpha_1$  adrenoceptor activation effectively primes excitatory synapses that terminate on the magnocellular neuroendocrine cells of the PVN. Physiologically, this interaction between two distinct classes of presynaptic metabotropic receptors ( $\alpha_1$  adrenoceptor and group III mGluR) may provide a means by which hormone output can be sustained or potentiated to meet homeostatic demands.

### $\alpha_1$ Adrenoceptor-mediated activation of PKC increases glutamate release

We demonstrate here that activation of PKC by  $\alpha_1$  adrenoceptors increases glutamate release from excitatory afferent fibers in PVN. This may result from distinct actions of this kinase at different steps in the release process, including the mobilization of neurotransmitter-filled vesicles from the reserve pool (Gillis et al., 1996; Stevens and Sullivan, 1998), an increase in the sensitivity of the release process for  $\text{Ca}^{2+}$  (Brager et al., 2002), and the facilitation of vesicle fusion (Scepek et al., 1998; Yawo, 1999). One, or a combination, of these mechanisms likely underlies the initial NA-induced increase in mEPSC frequency.

We also observe an increase in the amplitude of mEPSCs in response to the second application of NA. Changes in the amplitude of miniature events are normally associated with postsynaptic changes in receptor numbers (Conti and Weinberg, 1999). Accordingly, the increase in event amplitude may result from an interaction between postsynaptic  $\alpha_1$  adrenoceptors and AMPA receptors, although studies examining a link between PKC and

postsynaptic AMPA receptors suggest a depression of AMPA receptor function after PKC activation (Daw et al., 2000). Another possibility is that mEPSCs observed in response to the first application of NA act as trophic factors to increase postsynaptic spine density (McKinney et al., 1999) and thus may explain the increase in amplitude in response to the second application of NA. This effect, however, occurs over a much longer time frame, requiring many hours to develop. An alternate possibility is that changes in amplitude are conferred presynaptically. Excitatory synapses onto magnocellular neurons of the SON exhibit multiquantal release when the probability of release is elevated by high-frequency trains (Kombian et al., 2000). Our finding that mEPSC amplitude increases when group III mGluRs are blocked suggest that increases in amplitude in response to the second application of NA (when mGluRs are inactive) may be controlled presynaptically. The recent description of increases in spontaneous EPSC amplitude after inhibition of presynaptic mGluRs in the sensorimotor cortex (Bandrowski et al., 2003) is consistent with this idea.

### PKC inhibition of mGluR activity

In addition to the short-term increases in neurotransmitter release, increasing evidence suggests that protein kinases may also serve as molecular substrates that regulate the initiation and maintenance of longer-term changes in synaptic efficacy. Our findings demonstrate that PKC serves as a molecular link between  $\alpha_1$  adrenoceptors and group III mGluRs, leading to a long-lasting inhibition of mGluRs. Because these receptors play a pivotal role in gating excitatory input to magnocellular neurons (Schraeder and Tasker, 1997; Oliet et al., 2001), their functional inactivation by PKC results in an amplification of glutamate release when the synapses are rechallenged by a second application of NA. Our findings are supported by the demonstration that PKC interacts with the group II and group III mGluRs to inhibit their function (Macek et al., 1999; Sorensen et al., 2002). The inhibition by PKC of group III mGluRs may result from an uncoupling of the receptor from its G-protein cascade (Macek et al., 1999) or by a direct phosphorylation of Ser<sup>862</sup> on the intracellular C terminus by PKC (Sorensen et al., 2002). The demonstration that the endogenous ligand NA can inhibit group III mGluR function is consistent with previous findings from the hippocampus that adenosine analogs, which also increase PKC activity (Macek et al., 1998), inhibit mGluR function. Importantly, we now demonstrate that the inhibition of mGluRs conferred by PKC is long lasting, extending well beyond the time frame of  $\alpha_1$  adrenoceptor activation. Exposing synapses to NA once increases the capacity of the terminals to release glutamate in response to subsequent exposure to the agonist. Essentially, the synapses have been primed.

### Mechanism of mGluR inhibition of mEPSC release

Group III mGluRs can decrease neurotransmitter release by inhibiting presynaptic voltage-dependent calcium channels (Perroy et al., 2002). Because the TTX-insensitive release of glutamate in magnocellular neurons does not depend on extracellular  $\text{Ca}^{2+}$  (Inenaga et al., 1998), an interaction between mGluRs and presynaptic calcium channels is unlikely to be responsible for the effects observed here. Our findings, when coupled with the demonstration that glutamate release at mitral bulb synapses is also regulated by group III mGluRs in a manner that is downstream of  $\text{Ca}^{2+}$  influx (Schoppa and Westbrook, 1997), provide evidence for an alternative mechanism through which mGluRs may inhibit transmitter release. The demonstration that a subset of

group III mGluRs is located at the site of vesicle fusion (Shigemoto et al., 1996) suggests that the effect we described may be attributable to a direct protein–protein interaction between the mGluR complex and a part of the vesicle release machinery (Scanziani et al., 1995).

The role of mEPSCs in conveying information at CNS synapses has been hotly debated (for review, see Staley, 1999). Findings from a number of central nuclei suggest that these stochastic, action potential-independent events may not be mere noise. Increasing the frequency of mEPSCs has functional consequences for action potential generation in magnocellular neuroendocrine cells (Kombian et al., 2000) and cerebellar interneurons (Carter and Regehr, 2002). Additionally, a decrease in miniature GABAergic IPSCs has been linked in the hippocampus to the development of epilepsy (Hirsch et al., 1999). This increase in the background “noise” resulting from the activation of  $\alpha_1$  adrenoceptors may in fact lead to an increase in the general excitability of these cells.

### Physiological significance

Adaptive responses mediated by the hypothalamus generally require sustained activation until homeostasis is achieved. In an attempt to restore fluid homeostasis in response to acute challenges such as dehydration or hemorrhage, the magnocellular neuroendocrine cells exhibit a prolonged increase in firing rate (Wakerley et al., 1978). This increase in activity, in response to a single acute stimulus, may outlast the physiological perturbation by >24 hr in some cases (Wakerley et al., 1978). Furthermore, the second stimulus in a repeated hemorrhagic protocol elicits an increase in vasopressin release compared with the first stimulus (DeMaria et al., 1987; Lilly et al., 1989). Our findings that NA primes the excitatory synaptic input to magnocellular neuroendocrine cells offer a potential explanation for this observation. The synaptic mechanisms described here would act in concert with previously described changes in activity of these cells in response to changes in plasma osmolarity (Oliet and Bourque, 1993) to increase spiking activity.

Because mGluRs are vital in regulating the synaptic excitability of these cells (Schrader and Tasker, 1997; Oliet et al., 2001), it stands to reason that activating intracellular pathways that inhibit the activity of these receptors would provide an ideal solution for sustaining or potentiating glutamatergic input. That this switch may be initiated by NA, which is released in the hypothalamus during the response to physiological stressors, only serves to highlight the exceedingly clever design of neuronal circuitry that restores homeostasis.

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