CHAPTER 2

Modulation of hypothalamic NMDA receptor function by cyclic AMP-dependent protein kinase and phosphatases


ABSTRACT

In the present study we investigated the modulation of hypothalamic NMDA receptor-mediated currents by cAMP-dependent protein kinase (PKA) using the two electrode voltage clamp technique in *Xenopus* oocytes injected with rat hypothalamic mRNA. Application of forskolin, which activates PKA by means of cAMP stimulation, caused a transient increase of NMDA-induced currents, whereas the inactive forskolin analogue 1,9-dideoxyforskolin had no effect. Incubation of oocytes with a membrane-permeable analogue of cyclic AMP, 8-bromoadenosine 3',5'-cyclic monophosphate, potentiated NMDA responses even more prominently than with forskolin. NMDA-induced currents recorded from *Xenopus* oocytes injected with cRNA encoding the NMDA receptor subunits NR1, NR2A, and/or NR2B, mainly found in rat hypothalamus, were not affected by PKA activation but were increased by PKC stimulation. It is interesting that inhibition of endogenous protein phosphatase 1 and/or 2A by calyculin A resulted in a similar enhancement of hypothalamic NMDA-induced currents. Preinjection of oocytes with calyculin A impeded the PKA- but not the PKC-mediated potentiation of hypothalamic NMDA-induced currents. We propose the involvement of an additional third messenger in the PKA effect, which acts most likely via the inhibition of tonically active protein phosphatase 1 and/or 2A.
INTRODUCTION

The hypothalamus acts as a major regulatory center for autonomic and endocrine homeostasis. Structurally, it consists of a group of nuclei that regulate a broad array of physiological and behavioral activities, including feeding and metabolism but also emotional behavior and reproduction (Brann and Mahesh, 1994).

Glutamate, the main excitatory neurotransmitter in the central nervous system, is found in large concentrations in various important hypothalamic nuclei (Van den Pol et al., 1990; Van den Pol, 1991; Goldsmith et al., 1994) and is able to excite virtually all neurons tested in the hypothalamus, including the arcuate nucleus, the median eminence, and other medial hypothalamic regions (Arnauld et al., 1983; Van den Pol et al., 1990). Studies examining the effect of glutamate antagonists on spontaneous excitatory postsynaptic potentials in hypothalamic nuclei have clearly demonstrated that glutamatergic neurotransmission is an important component in the regulation of neuroendocrine function in the hypothalamus (Gribkoff and Dudek, 1990; Van den Pol et al., 1990; Waurin and Dudek, 1991; Inenaga et al., 1998).

The majority of studies investigating the NMDA receptor, an important ionotropic glutamate receptor subtype distributed throughout the hypothalamus (Van den Pol et al., 1994; Kus et al., 1995), have focused on its role in inducing release of hormones such as growth hormone and gonadotropin-releasing hormone, but it has also been demonstrated to play a pivotal role in the induction and regulation of the circadian rhythm (reviewed in: Brann and Mahesh, 1994).

Although the importance of the NMDA receptor in regulating the hypothalamic neuroendocrine system is now widely acknowledged, little is known about the modulation of hypothalamic NMDA receptor function itself. In general, a common mechanism for the modulation of the NMDA receptor-channel complex is phosphorylation by second messenger-dependent protein kinases.

In the present study, we investigated the modulation of hypothalamic NMDA receptor function by cyclic AMP (cAMP)-dependent protein kinase (PKA) and its possible underlying mechanism by using the two electrode voltage clamp technique in *Xenopus* oocytes.

MATERIALS AND METHODS

Materials

Mature female specimens of *Xenopus* laevis were obtained from Kähler (Hamburg, Germany). Collagenase (type II) was from Worthington Biochemicals (Freehold, NJ, U.S.A.). The mRNA Purification Kit was from Pharmacia LKB Biotechnology (Uppsala, Sweden). Calyculin A (Cal A) was from Boehringer (Mannheim, Germany). 1,9-Dideoxyforskolin, 8-bromoadenosine 3’5’-cyclic monophosphate (8-Br-cAMP; sodium salt) and H-89 dihydrochloride were from Calbiochem (La Jolla, CA, U.S.A.). Phorbol 12-myristate 13-acetate (PMA) and 4α-PMA were from Research Biochemicals (Natick, MA, U.S.A.). Penicillin and streptomycin were from Gibco (Paisley, U.K.). All other drugs and salts were purchased from Sigma (St. Louis, MO, U.S.A.).
We confirm that all animal protocols used have been approved by the animal experimentation committee of the State Government of Lower Saxony.

Handling, Injection, and Maintenance of Oocytes

Oocytes were surgically obtained from adult *X. laevis* frogs that were anesthetized by immersion into 3-aminobenzoic acid ethyl ester (1 g/L) for 45 min. Follicular cell layers were removed manually after incubation in Ca2+-free modified Barth’s solution [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 15 mM HEPES, 0.8 mM MgSO4, 50 U/ml penicillin and 50 µg/ml streptomycin (adjusted to pH 7.6 with NaOH)] containing 3.0 mg/ml collagenase (type II) for 2 h at room temperature (20-24°C). Stage V and stage VI oocytes were pressure-injected with 50 ng hypothalamic poly (A)+ mRNA or 10-15 ng NR1b cRNA within 24 h after harvesting. In some experiments the NR1b cRNA was co-injected with NR2A and/or NR2B cRNA (25-30 ng) in a 1:2 ratio. After injection, the oocytes were maintained at 18°C in modified Barth’s solution. Modified Barth’s solution contained 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 15 mM HEPES, 0.8 mM MgSO4, 0.3 mM Ca(NO3)2, 0.6 mM CaCl2, 50 U/ml penicillin and 50 µg/ml streptomycin (adjusted to pH 7.6 with NaOH).

RNA Preparation

Total RNA was isolated from the hypothalamus of adult Wistar rats by extraction of fresh tissue with guanidine thiocyanate and precipitation with LiCl (Cathala et al., 1983). Poly(A)+ mRNA was purified by oligo(dT) cellulose chromatography (Pharmacia mRNA Purification Kit) and dissolved in RNase-free water at a concentration of 0.5 µg/µl.

Plasmids containing the cDNA clones encoding the NR1b, NR2A, and NR2B subunits were linearized by digestion with *Pvu*I (NR1b) or *Not*I (NR2A and NR2B) and used as transcription templates. *In vitro* transcription was carried out by T7 or T3 RNA polymerase in the presence of the capping reagent m 7G(5')ppp(5')G. RNA transcripts were precipitated by ethanol, and the precipitate was dissolved in diethyl pyrocarbonate-treated water.

Electrophysiological Recordings

At 7-8 days after injection oocytes were placed in a recording chamber (volume = 20 µl) and continuously superfused (1.0 ml/min) with recording solution at room temperature. The recording solution contained (115 mM NaCl, 2.5 mM KCl, 1.8 mM BaCl2 and 10 mM HEPES (pH adjusted to 7.2 with NaOH). The oocytes were voltage-clamped by a conventional two-microelectrode voltage-clamp technique (Stühmer, 1992). The membrane potential of the oocytes was held at -80 mV using a Turbo Clamp Tec 01C amplifier (N.P.I. Electronic, Tamm, Germany). The two microelectrodes were filled with 3 M KCl and had resistances between 1-3 MΩ. NMDA currents were elicited by switching the flow from recording solution to recording solution containing NMDA (100 µM) and glycine (10 µM) without changing the perfusion rate.

In some experiments oocytes were preincubated with 8-Br-cAMP (10 mM) or Cal A (100 nM). During this incubation oocytes were continuously superfused with recording solution containing the drug to be applied. In some experiments, oocytes were incubated for 3 h with H-89, which was diluted to a final concentration of 10 µM in modified Barth’s solution. The H-89 stock solution (10 mM) was prepared in dimethyl sulfoxide (DMSO). Forskolin (50 µM), 1,9-dideoxyforskolin (50 µM), PMA (10 nM), and 4α-PMA (10 nM) were steadily applied for 1 min with a micropipette (200 µl) after stopping the solution flow. Cal A was prepared in 100% ethanol as 1 mM stock solution, which was diluted with recording solution to the appropriate concentration shortly before the electrophysiological experiments. Cal A was prepared in 100% ethanol as 1 mM stock solution, which was diluted with recording solution to the appropriate concentration shortly before the electrophysiological experiments. Cal A was preinjected 50 - 70 min before voltage-clamping following the RNA injection protocol. Aliquots of concentrated stock solutions of forskolin, 1,9-dideoxyforskolin, PMA and 4α-PMA in DMSO and stock solutions of NMDA and glycine in distilled water were added to the recording solution immediately before the experiments. The final DMSO concentration never exceeded 0.1% (vol/vol), which, upon application, induced no change in membrane current by itself and which had no effect on NMDA-induced currents in control experiments. 8-Br-cAMP was directly dissolved to the final concentration in the recording solution. Effects of the various compounds were tested after at least two or three identical responses to NMDA could be elicited (control responses). Oocytes were not used for experiments if reproducible responses to NMDA could not be recorded within 30 min.

Current signals were low-pass-filtered at 30 Hz using a four-pole Bessel filter and digitized by an ITC 16-MAC interface (Instrutech Corp., Great Neck, NY). Data were sampled at 100 Hz and stored on a Macintosh (7100/66) computer using data acquisition software (Pulse 7.40; HEKA...
Electronic, Lambrecht/Pfalz, Germany). To compare responses between different oocytes, the amplitude of the NMDA-induced current at any given time was normalized with respect to the amplitude of control responses. The n value represents the number of oocytes in one experiment. At least two different batches of oocytes were used per experiment.

Statistics
Statistical comparisons were made using Student's t test. Results were expressed as mean ± SEM values. Values of p ≥ 0.05 were regarded as not significant.

RESULTS
Stimulation of cAMP potentiates hypothalamic NMDA responses
The effect of cAMP stimulation on NMDA-induced responses was examined in oocytes injected with hypothalamic mRNA isolated from adult rat brain. Application of NMDA together with glycine at a holding potential of -80 mV elicited responses with the typical characteristics previously described for NMDA-induced responses (Leonard and Kelso, 1990; Kelso et al., 1992). The average response amplitude was 25 ± 6 nA (n = 34).

Figure 1. Enhancement of hypothalamic NMDA-induced responses by cAMP stimulation. A. Representative NMDA responses of hypothalamic mRNA-injected oocytes before (control) and after 1-min application of 50 µM forskolin or 10-min application of 10 mM 8-Br-cAMP. B and C. Time course of NMDA-induced current amplitudes after application of 50 µM forskolin or 50 µM 1,9-dideoxyforskolin (B) or (C) 10 mM 8-Br-cAMP. Horizontal bars intervals of drug superfusion. External solution was applied in control experiments. Data are the mean ± SEM (bars) values of three to five oocytes.
When oocytes were incubated with forskolin (50 µM), which activates PKA by increasing cAMP levels (reviewed by Seamon and Daly, 1986), an enhancement of the NMDA response amplitude was observed with a maximum reached after 7 min (150 ± 9%; n = 5; Fig. 1A and B). This forskolin concentration appeared to be the minimal concentration required to exhibit maximal forskolin effects on NMDA-induced currents (data not shown). Because it is known that forskolin can sometimes act directly on ion channels without the involvement of cAMP (Laurenza et al., 1989), oocytes were incubated with 1,9-dideoxyforskolin (50 µM), an inactive analogue of forskolin that does not activate adenylate cyclase. 1,9-Dideoxyforskolin did not affect NMDA responses (100 ± 0%; n = 3; Fig. 1B), indicating that forskolin caused the potentiation of NMDA-induced responses via elevated cAMP levels. Bath application of 8-Br-cAMP (10 mM, 10 min), a membrane-permeable analogue of cAMP, also caused a large and transient increase of hypothalamic NMDA responses, which was more prominent than the forskolin effect (225 ± 19%; n = 3; Fig. 1A and C). Repeated application of NMDA and glycine with 2-min intervals showed no change in NMDA response amplitude for the time course of the experiments (indicated as control; Fig. 1).

Stimulation of cAMP does not affect responses of recombinant NMDA receptors

To test whether PKA potentiates NMDA-induced currents via a direct action on the NMDA receptor itself, we investigated the effect of forskolin (50 µM) incubation on NMDA-induced currents recorded from oocytes expressing recombinant NMDA receptors.

Table 1. Effect of protein kinase modulators on recombinant NMDA receptors

<table>
<thead>
<tr>
<th>Compound</th>
<th>NR subunits*</th>
<th>1b</th>
<th>1b/2A</th>
<th>1b/2B</th>
<th>1b/2A2B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>1.02 ± 0.03 (3)</td>
<td>0.98 ± 0.07 (3)</td>
<td>0.87 ± 0.09 (3)</td>
<td>1.04 ± 0.08 (3)</td>
</tr>
<tr>
<td>Forskolin (50µM)</td>
<td></td>
<td>1.11 ± 0.05 (5)</td>
<td>1.01 ± 0.07 (3)</td>
<td>0.95 ± 0.02 (3)</td>
<td>1.01 ± 0.06 (3)</td>
</tr>
<tr>
<td>PMA (10 nM)‡</td>
<td></td>
<td>2.08 ± 0.28 (6)</td>
<td>2.13 ± 0.38 (3)</td>
<td>1.68 ± 0.16 (5)</td>
<td>1.69 ± 0.30 (3)</td>
</tr>
<tr>
<td>4-α-PMA (10 nM)‡</td>
<td></td>
<td>0.99 ± 0.03 (3)</td>
<td>0.85 ± 0.19 (3)</td>
<td>0.89 ± 0.11 (3)</td>
<td>0.87 ± 0.08 (3)</td>
</tr>
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</table>

* Data are mean ± SEM values of the relative current amplitude as determined by the \( I/I_c \) ratio of steady-state responses to 100 µM NMDA and 10 µM glycine after (I) and before (I_c) 1-min application of recording solution (control), 50 µM forskolin, 10 nM PMA, or 10 nM 4α-PMA. The number of oocytes in an experiment is given in parentheses. Experiments were performed at a holding potential of -80 mV.
We recorded NMDA-induced currents from oocytes injected with cRNA encoding NR1b, NR1b/NR2A, NR1b/NR2B, and NR1b/NR2A/NR2B subunits, the main subunits found in the adult rat hypothalamus (Laurie et al., 1997). Average response amplitudes were 36.8 ± 3.0 nA (n = 20) for oocytes expressing NR1b, 476 ± 207 nA (n = 15) for oocytes expressing NR1b/NR2A, 13,390 ± 2,389 nA (n = 17) for oocytes expressing NR1b/NR2B, and 21,847 ± 7,354 nA (n = 15) for oocytes expressing NR1b/NR2A/NR2B. In contrast to the potentiation of NMDA-induced current amplitudes recorded from oocytes injected with hypothalamic mRNA, incubation with forskolin did not affect NMDA-induced currents from recombinant NMDA receptors (Table 1). However, a 1-min incubation with PMA (10 nM), a potent protein kinase C (PKC) activator, resulted in a transient potentiation of the NMDA-induced current responses. This potentiation reached a maximum value after 17 min (Table 1). The inactive PMA analogue 4α-PMA (10 nM) had no effect (Table 1).

Inhibitors of PKA or protein phosphatases prevent forskolin-mediated potentiation of hypothalamic NMDA-induced currents

Because NMDA-induced responses can not only be modulated by protein kinases but also by endogenous protein phosphatases, we studied the effect of protein phosphatase inhibition on hypothalamic NMDA-induced currents. When oocytes injected with hypothalamic mRNA were incubated with Cal A (100 nM, 5 min), a potent inhibitor of protein phosphatase 1 (PP1) and/or 2A (PP2A) (Ishihara and Martin, 1989; Suganuma et al., 1990), the NMDA-induced current amplitude increased to 159 ± 4% (n = 3) and reached a maximal potentiation after 50 - 70 min (Fig. 2). The potentiation of hypothalamic NMDA responses observed after application of Cal A did not significantly differ from the value obtained after forskolin application.

Next, we were interested in whether forskolin could still enhance NMDA responses after Cal A treatment. To circumvent run-down problems during electrophysiological recordings over several hours, oocytes were preinjected instead of incubated with Cal A to a final intracellular concentration of 500 nM 50 - 70 min before voltage voltage-clamping. Subsequent incubation with forskolin could no
longer enhance NMDA-induced currents (99 ± 1%; n = 5; Fig. 3A). The forskolin-mediated potentiation could also be blocked (99 ± 0.7%; n = 5; Fig. 3A) by preincubation of oocytes with a cell-permeable, selective, potent inhibitor of PKA, H-89 (10 µM, 3 h). A 1-min incubation with PMA (10 nM) also increased NMDA-induced current amplitudes from oocytes injected with hypothalamic mRNA. This potentiation reached a maximum 17 min after the start of the incubation. Preinjection of oocytes with Cal A (500 nM) had no significant effect on the PMA-mediated potentiation (Fig. 3B).

**Figure 3.** Cal A antagonizes PKA- but not PKC-mediated potentiation of hypothalamic NMDA responses. The time course of NMDA-induced current amplitudes was recorded from oocytes injected with hypothalamic mRNA with or without preinjection of Cal A to a final intracellular concentration of 500 nM 50-70 min before voltage clamping. The intracellular concentration of Cal A was calculated by assuming standard oocytes with a volume of 0.5 µl. The horizontal bar indicates a 1-min drug application of either (A) control solution or forskolin (50 µM) or (B) control solution or PMA (10 nM). Some oocytes were incubated for 3 h in 10 µM H-89 before forskolin application (A). Data are mean ± SEM (bars) values of three to five oocytes.

**DISCUSSION**

In the present study we investigated the modulation of hypothalamic NMDA receptors by PKA and PP1 and/or PP2A. NMDA-induced currents recorded from oocytes injected with rat hypothalamic mRNA were increased after stimulation of PKA, whereas PKA stimulation had no significant effect on NMDA currents from recombinant NMDA receptors. Recently, Leonard and Hell (1997) demonstrated that PKA and members of the PKC family phosphorylate all three NMDA receptor subunits in vitro and in vivo. The main phosphorylation sites of PKA and PKC are substantially different for NR1, NR2A, and NR2B in agreement with the finding that
phosphate incorporation by PKA and PKC is additive. However, both kinases are equally efficient in phosphorylating NR1 in vitro (Leonard and Hell, 1997). Thus, the question arises why NMDA currents in oocytes expressing NMDA receptor subunits were not sensitive to forskolin. It is highly unlikely that forskolin did not enhance NMDA responses of recombinant NMDA receptors simply because the receptors were already fully phosphorylated and could therefore not produce any further enhancement because PKC stimulation could still potentiate NMDA-induced currents from recombinant receptors in our study. Moreover, Xenopus oocytes have been shown to express endogenously all necessary components to activate PKA effectively (Keller et al., 1992). Therefore, it does not seem probable that forskolin was not efficient in stimulating PKA when cRNA coding for receptor subunits was injected instead of hypothalamic mRNA.

Although the NMDA receptor was shown to have distinct PKA and PKC phosphorylation sites, only the forskolin-mediated enhancement of hypothalamic NMDA responses was prevented by pretreatment with the general PP1 and PP2A inhibitor Cal A. PMA was still able to enhance NMDA responses after Cal A treatment. In agreement with our data obtained using the Xenopus oocytes expression system, Westphal et al. (1999) found that responses recorded from HEK 293 cells expressing heteromeric NMDA receptors consisting of NR1a and NR2A are also barely susceptible to activation of the PKA system. However, this enhancement is intensified if yotiao, an NMDA receptor-associated protein that can bind PP1 and the PKA holoenzyme, is coexpressed with NMDA receptor subunits. It is interesting that anchored PP1 is active and thereby tonically inhibits nearby NMDA receptors (Westphal et al., 1999). This finding is in support of our observation that inhibition of protein phosphatase activity by Cal A enhanced hypothalamic NMDA responses. Westphal et al. (1999) further demonstrated that, in the presence of yotiao, PKA activation overcomes the tonic PP1 activity, causing rapid enhancement of NMDA receptor currents. We might not have observed any further forskolin effect after Cal A treatment because the Cal A application already completely shifted the equilibrium from dephosphorylation to phosphorylation by the inhibition of PP1.

So far, all electrophysiological experiments investigating the modulation of NMDA responses by PKA activation documented a clear brain region-specific interaction. In previous studies, we demonstrated that NMDA responses recorded from oocytes injected with total rat brain, hippocampal, or cortical mRNA were not affected by incubation with forskolin (Blank et al., 1996; 1997; T.B. and I.N., unpublished data). Similarly, whole-cell recordings with the standard patch-clamp method showed that forskolin had no effect on current responses to NMDA in cultured hippocampal pyramidal neurons (Greengard et al., 1991). On the other hand, stimulation of PKA enhances NMDA responses, for example, in rat spinal dorsal horn neurons (Cerne et al., 1993) and rat neostriatal neurons (Colwell and Levine, 1995).

However, these studies did not clearly identify the target substrate of PKA action. Previously, we showed the involvement of another subcellular signaling molecule, the 32-kDa dopamine- and cAMP-regulated phosphoprotein (DARPP-32), in cAMP-dependent modulation of striatal NMDA responses (Blank et al., 1997). Like yotiao, DARPP-32 shifts the equilibrium in favor of phosphorylation following
PKA modulation of hypothalamic NMDA responses

PKA stimulation. Future studies have to determine which signaling molecule might be involved in the PKA-mediated potentiation of hypothalamic NMDA responses. A possible candidate is the phosphoprotein inhibitor-1, which has a high sequence homology with DARPP-32 and was shown to be abundant in several hypothalamic nuclei (Gustafson et al., 1991). Like DARPP-32, inhibitor-1 is converted from an inactive form to a potent and specific inhibitor of PP1 by PKA phosphorylation (Hemmings et al., 1992).

Our finding that Cal A did not significantly lower the effectiveness of PMA to enhance NMDA currents points to a different underlying mechanism of PKC-mediated potentiation than overcoming endogenous protein phosphatase activity. Recent studies have indicated that calmodulin binds directly to the NR1 subunit of the NMDA receptor complex at two distinct sites in the COOH-terminal region, causing a decrease in NMDA channel activity by reduction of the probability of channel opening (Ehlers et al., 1996). PKC-mediated phosphorylation on serine residues of the NR1 subunit decreases its affinity for calmodulin and thereby inhibits the inactivation of NMDA receptors (Hisatsune et al., 1997). Because calmodulin is an ubiquitous molecule that is also enriched in *Xenopus* oocytes (Lan et al., 1996; Peracchia et al., 1996), it is conceivable that PKC, in contrast to PKA, potentiates currents evoked from both recombinant NMDA receptors and hypothalamic NMDA receptors by reducing the affinity of calmodulin to NR1.

This study provides evidence that the function of hypothalamic NMDA receptors can be regulated by PKA and suggests the involvement of an additional NMDA receptor-associated molecule that acts most likely via the inhibition of tonically active protein phosphatases. Thus, several PKA-coupled neurotransmitter systems might use this cascade in the rat hypothalamus to interfere with the signaling of NMDA receptors.

ACKNOWLEDGMENTS

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42


