Metabotropic Glutamate Receptor Modulation of Glutamate Responses in the Suprachiasmatic Nucleus

LAUREL L. HAAK
Neurosciences Program, Department of Biological Sciences, Stanford University, Stanford, California 94305-5020

Haak, Laurel L. Metabotropic glutamate receptor modulation of glutamate responses in the suprachiasmatic nucleus. J. Neurophysiol. 81: 1308–1317, 1999. Glutamate is the primary excitatory transmitter in the suprachiasmatic nucleus (SCN). Ionotropic glutamate receptors (iGluRs) mediate transduction of light information from the retina to the SCN, an important circadian clock phase-shifting pathway. Metabotropic glutamate receptors (mGlurRs) may play a significant modulatory role. mGluR modulation of SCN responses to glutamate was investigated with fura-2 calcium imaging in SCN explant cultures. SCN neurons showed reproducible calcium responses to glutamate, kainate, and N-methyl-D-aspartate (NMDA). Although the type I/II mGluR agonists L-CCG-I and t-ACPD did not evoke calcium responses, they did inhibit kainate- and NMDA-evoked calcium rises. This interaction was insensitive to pertussis toxin. Protein kinase A (PKA) activation by 8-bromo-cAMP significantly reduced iGluR responsivity in SCN cells. About one-half of the calcium rise evoked by enhanced by activating protein kinase C (PKC) and significantly (PKA) activation by 8-bromo-cAMP significantly reduced iGluR inhibition. This interaction was insensitive to pertussis toxin. Protein kinase A (PKA) activation by 8-bromo-cAMP significantly reduced iGluR inhibition by mGluR agonists. The inhibitory effect of mGluRs on calcium rises was enhanced by activating protein kinase C (PKC) and significantly reduced in the presence of the PKC inhibitor H7. Previous reports show that L-type calcium channels can be modulated by PKC and PKA. In SCN cells, about one-half of the calcium rise evoked by kainate or NMDA was blocked by the L-type calcium channel antagonist nimodipine. Calcium rises evoked by K+ were used to test whether mGluR inhibition of iGluR calcium rises involved calcium channel modulation. These calcium rises were primarily attributable to activation of voltage-activated calcium channels. PKC activation inhibited K+-evoked calcium rises, but PKC inhibition did not affect L-CCG-I inhibition of these rises. In contrast, 8Br-cAMP had no effect alone but blocked L-CCG-I inhibition. Taken together, these results suggest that activation of mGluRs, likely type II, modulates glutamate-evoked calcium responses in SCN neurons. mGluR inhibition of iGluR calcium rises can be differentially influenced by PKC or PKA activation. Regulation of glutamate-mediated calcium influx could occur at L-type channels, K+ channels, or at GluRs. It is proposed that mGluRs may be important regulators of glutamate responsivity in the circadian system.

INTRODUCTION

Animals exhibit daily, or circadian, rhythms generated by an endogenous oscillator located in the suprachiasmatic nucleus (SCN), which are bilateral hypothalamic nuclei located above the optic chiasm. This oscillator functions in the absence of external time cues but can be entrained by environmental stimuli such as the light–dark cycle. Photic information is carried to the SCN via the retinohypothalamic tract (RHT) (Johnson et al. 1988; Moore and Lenn 1972; Pickard 1982; Speh and Moore 1993). Stimulation of the RHT releases glutamate onto SCN cells (Cahill and Menaker 1989; Liou et al. 1986). Besides glutamate, several transmitters and modulators are found in the SCN (van den Pol and Tsujimoto 1985), some of which can directly modulate the effects of light. In addition to the RHT, light influences the SCN via the neuropeptide Y (NPY)-containing geniculohypothalamic tract (Morin and Blanchard 1995). NPY activates protein kinase A (PKA) (Grouzmann et al. 1997; Sherriff et al. 1997) and has long-term effects on glutamatergic activity in SCN neurons in vitro (van den Pol et al. 1996). NPY can also phase shift the circadian clock (Ding et al. 1998; Huhman and Albers 1994; Medanic and Gillette 1993; Park et al. 1997). In addition, the effects of light on the circadian system are modulated by melatonin (Cassone 1992). Both PKC and cAMP are implicated in the phase-resetting effects of melatonin (Zatz 1989; McArthur et al. 1997). Therefore second messengers activated by modulators such as NPY and melatonin may interact with GluR-activated transduction pathways and alter photic responsiveness of SCN cells. Investigation of the influence of the second messengers protein kinase C (PKC) and PKA on glutamate responses in SCN cells may indicate how these multiple pathways interact.

The SCN contains both iGluRs (Ebling 1996; Gannon and Rea 1994; Mikkelsen et al. 1993, 1995; Stamp et al. 1997; van den Pol et al. 1994a) and mGluRs (Gannon and Rea 1994; Ghosh et al. 1997; Mick et al. 1995; van den Pol 1994; van den Pol et al. 1994, 1995). iGluRs are involved in transducing photic information; iGluR antagonists can block the effects of light, and iGluR agonists can mimic the effects of light (Cahill and Menaker 1987, 1989; Ding et al. 1994; Kim and Dudek 1991; Mintz and Albers 1997). Although it appears that glutamate and GluRs are critical for clock entrainment to light, the cellular transduction pathway involved and how it may be modulated are not yet clear. In situ calcium imaging indicates that RHT stimulation evokes calcium rises in specific SCN cells and may involve both iGluRs and mGluRs (Tomina et al. 1994).

Interactions between GluR subtypes and consequent regulation of intracellular calcium levels were demonstrated in several systems. mGluRs were linked to modulation of iGluRs (Dildy-Mayfield and Harris 1994; Kelso et al. 1992) and calcium entry (Gereau and Conn 1995; Sahara and Westbrook 1993; Swartz and Bean 1992). In several systems, mGluR inhibition of glutamatergic transmission is modulated by PKC (Croxtall et al. 1997; Swartz et al. 1993; Tyler and Lovinger 1995). Specific mGluR agonists stimulate translocation of PKC from the cytosol to the membrane (Fukunaga et al. 1992; Speh and Moore 1993). Stimulation of the RHT releases glutamate onto SCN cells (Cahill and Menaker 1989; Liou et al. 1986).
Vaccarino et al. 1991), where it plays a critical role in signal transduction, including phosphorylation of voltage-activated calcium channels (VACCs) (Stamp et al. 1997; Zamponi et al. 1997). L-type channel activity in Xenopus oocyte expression systems and in acutely dissociated neurons can be inhibited by PKC (Doerner et al. 1988, 1990; Sena et al. 1995; Singer-Lehat et al. 1992) or by PKA (Armstrong and Eckert 1987; Perez-Reyes et al. 1994).

mGluRs are divided into three groups based on sequence homology of the cloned receptors, pharmacology, and second messenger pathways (Nakanishi and Masu 1994). Type I and II mGluRs were implicated in PKC activation (Maiese et al. 1996). Activation of type II and III mGluRs inhibits adenylate cyclase in several systems. mGluR activation may be an important means of regulating SCN responsiveness to glutamate. Previous experiments in isolated SCN astrocytes have shown that short-term application of mGluR agonists causes a long-lasting inhibition of iGluR-evoked calcium rises (Haak et al. 1997a).

Multiple mGluRs are expressed in the SCN, and activation of multiple transduction pathways by a single mGluR (Aramori and Nakanishi 1992) suggest there may be multiple coupling mechanisms and systems likely reflect the diverse physical functions regulated by calcium, from transmitter release to cytoskeletal stability to gene transcription.

It seems clear that glutamate plays a major role in entraining the circadian clock to external light cycles. In vivo experiments indicate that activation of iGluRs is necessary for light-induced clock phase shifting. However, little is known about the cellular transduction pathway that mediates this response or how it may be modulated. Is calcium involved? What second messengers are activated? Can the iGluRs be modulated? To shed some light on these questions, modulation of glutamate responses was investigated in SCN explant cultures with fura-2 digital calcium imaging. iGluR agonists evoked calcium rises in neurons, likely through the opening of L-type calcium channels (VACCs). iGluR-evoked calcium responses were inhibited by type II mGluR agonists. Part of this inhibition may be attributable to inhibition of VACCs. Evidence is provided suggesting that PKA and PKC influence the iGluR–mGluR interaction.

Some of these data were published previously (Haak 1997; Haak et al. 1997b).

METHODS

Tissue culture

SCN punches were prepared as described by Haak et al. (1997a). Briefly, brains were removed from neonatal (P1–P2) Sprague-Dawley rats (Simonsen, Gilroy, CA) and then washed three times in ice-cold Hank’s balanced salt solution (HBSS, GibcoBRL, Grand Island, NY). Animal care was in accordance with Stanford University Animal Care and Use Committee guidelines. Brains were placed ventral side down on a Teflon plate, and coronal slices (525 μM) were prepared with an automated tissue chopper. Slices were transferred to ice-cold HBSS. The bilateral SCN were punched from each slice with a polished 20-gauge stainless steel needle. Tissue punches were placed individually into 0.5-ml thin-walled microcentrifuge tubes containing 100 μl cold HBSS and washed twice with 100 μl HBSS lacking calcium and magnesium (HBSS-). Punches were incubated overnight at 4°C in 100 μl HBSS containing 0.2 × trypsin-EDTA (GibcoBRL). The next morning, punches were placed in a 37°C incubator for 30 min to activate the trypsin. Then the trypsin solution was removed, and 100 μl warm growth medium was added. Each punch was triturated very gently approximately five times, just until the tissue began to come apart. These barely dispersed punches were plated on 22 mm poly-D-lysine coated glass coverslips, each punch in a single well of a six-well tissue culture plate. Coverslips were previously soaked over-night in Cleaning Concentrate (Bio-Rad, Richmond, CA), rinsed for 1 h in deionized water, and then dried and autoclaved. To enhance attachment to the coverslip, dispersed punches were plated within a 7-mm-diam glass cloning ring placed on the coverslip. Three hours after plating, 2 ml growth medium was added per well, and rings were removed. Cultures were housed in a Napco 6100 incubator at 37°C and 5% CO2 and maintained on growth medium: glutamate- and glutamine-free MEM (GibcoBRL) supplemented with heat-inactivated 10% bovine calf serum (Hyclone, Logan, UT), 100 U/ml penicillin/streptomycin (GibcoBRL), and 1 mM kynurenate (RBI, Natick, MA) (Finkbeiner and Stevens 1988). Growth medium was replaced twice weekly. Cells were used after 8–10 days in vitro.

Punch immunocytochemistry

SCN punch explant cultures contained both neurons and astrocytes, as demonstrated by MAP2 and GFAP immunostaining (Fig. 1, A and B). During experiments, fura-2-loaded neurons were easily identified in punch cultures as bright cells with compact cell bodies (Fig. 1C). Approximately 95% of cells matching this description responded to N-methyl-D-aspartate (NMDA), further supporting their identity as neurons.

Immunostaining was performed as described by Haak et al. (1997a). Punches attached to coverslips were rinsed once in 0.1 M PBS (containing, in g/l, 8 NaCl, 0.2 KCl, 1.44 Na2HPO4, and 0.24 KH2PO4) and then permeabilized by incubating with ice-cold anhydrous methanol for ≥20 min at −20°C. After fixing, cells were rehydrated with four gentle rinses in PBS and then stained following the Vectastain Elite ABC Kit protocol (Vector Labs, Burlingame, CA). Cells were incubated with 1.5% goat serum diluted in PBS. After 30 min, serum was removed, and mouse monoclonal primary antibody diluted in 1.5% goat serum was incubated overnight at 4°C. After washing with three rinses of PBS, at which time the secondary goat anti-mouse antibody was added for 30 min. Then cells were washed with three rinses of PBS, incubated with a solution of avidin–biotin–peroxidase complex for 30 min, and again washed with PBS three times. Finally, staining was visualized with DAB intensified with NiCl2, added to the cells for 1–2 min. Cells were rinsed in PBS to stop the peroxidase reaction.

Calcium digital imaging

Punch cultures were loaded for 30 min at 37°C with 5 μM fura-2 acetoxyethyl ester (Molecular Probes, Eugene, OR) in a HEPES-buffered perfusion solution (containing 137 mM NaCl, 25 mM glucose, 5 mM KCl, 1 mM MgCl2, 3 mM CaCl2, and 10 mM HEPES, pH 7.4). After loading, cells were washed of excess fura-2, and a 180-μl laminar flow perfusion chamber was assembled (Forscher et al. 1987). HEPES buffer was perfused across the chamber at a constant rate of 1 ml/min. For experiments using zero- or low-magnesium HEPES buffer, the MgCl2 concentration was adjusted to

Downloaded from http://jn.physiology.org/ by IP 10.220.33.6 on May 9, 2017
with complete washout in Solutions moved uniformly and rapidly across the perfusion chamber, tions was accomplished with stopcock valves on the individual lines. that abutted the laminar flow cell surface. Switching between solu-
reservoir was connected to the flow cell by an individual capillary line frozen stocks and held in 50-ml reservoirs at room temperature. Each drug solutions were freshly prepared or diluted in HEPES buffer from zero or 250 \( \mu M \), respectively, and 2 \( \mu M \) glycine was added. Each day, drug solutions were freshly prepared or diluted in HEPES buffer from frozen stocks and held in 50-ml reservoirs at room temperature. Each reservoir was connected to the flow cell by an individual capillary line that abutted the laminar flow cell surface. Switching between solutions was accomplished with stopcock valves on the individual lines. Solutions moved uniformly and rapidly across the perfusion chamber, with complete washout in \( \sim 5 \) s. All experiments were performed at room temperature.

Cells were imaged on a Nikon Diaphot 300 inverted microscope with a \( \times 40 \) Olympus DApo objective with high UV transmittance. Individual cells were tagged with a \( 4 \times 4 \) pixel square. Between 20 and 50 cells could be recorded per experiment; 340/380-nm Ca\(^{2+} \) ratio images were captured every 2 s and stored to a 486 PC disk with FLUOR software (Universal Imaging, West Chester, PA). A Sutter (Novato, CA) Lambda 10 controller and filter wheel switched between 340- and 380-nm wavelengths. A 150-W xenon lamp (Optiquip, Highland Mills, NY) provided the excitation light, which was attenuated by 90% to minimize photobleaching and maximize recording time. Fluorescence emitted by fura-2 was passed through a 480-nm filter and into a Hamamatsu 2400 silicon-intensified target video camera. Ratiometric fluorescent values collected in this way were converted to Ca\(^{2+} \) concentrations based on a calibration curve established with fura-2 and calcium standard solutions from Molecular Probes (Gryniewicz et al. 1985). Calibrated Ca\(^{2+} \) data were analyzed on an Apple Macintosh Quadra 950 computer with IgorPro Software (WaveMetrics, Lake Oswego, OR). In the figures presented, each trace represents the ratiometric calcium response of one cell.

Data for histograms representing percentage change in evoked response were calculated by subtracting from the peak evoked Ca\(^{2+} \) rise from an averaged baseline Ca\(^{2+} \) level from the 3 min just before the Ca\(^{2+} \) rise. Increases of \( >60 \) nM over baseline were pooled and averaged. To obtain the percentage change, peaks evoked during experimental treatments were compared with controls and then normalized by dividing by the control peak value and multiplying by 100. Data are from two to five experiments on different cultures and are presented as the percentage change \( \pm SE \). To determine statistical significance, a two-tailed Student’s or Welch’s \( t \)-test was used.

**Chemicals**

Sodium-glutamate, poly-t-lysine hydrobromide (MW \( >300,000 \)), calphostin C, 1-oleyl-2-acetyl-sn-glycerol (OAG), and 8-bromo-cAMP (8Br-cAMP) were purchased from Sigma (St. Louis, MO); kynurenic acid, \( \text{trans-(} \pm \text{-)}-1\text{-amino-1,3-cyclopentanedicarboxylate (t-ACPD)} \), kainate, pertussis toxin (PTx), phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), tamoxifen citrate, \( \omega\)-conotoxin GVIA, and nimodipine were purchased from RBI (Natick, MA); and \( \text{(2S,1S,2S)}\)-2-(carboxycyclopropyl)-glycine (L-CG-I) and \( \text{(} \pm \text{-)}-1\text{-}(5\text{-isoquinoline-sulfonyle})\)-2-methylpiperazine (H7 dihydrochloride) were pruchased from Tocris Cookson (St. Louis, MO).

**RESULTS**

**Glutamate responsiveness in SCN neurons**

GLUR AGONIST DOSE-RESPONSE CURVES. To determine the dose-response relationship for GluR agonists in SCN neurons, punch cultures were tested with a range of GluR agonist concentrations, applied twice for 20 s each. Only cells that responded to both agonist applications were included in the analysis for that dose. Each concentration was tested on at least three cultures, and results are expressed as percentage of maximum evoked calcium rise \( \pm SE \) (Fig. 2). The \( EC_{50} \) represents the dose at which the GluR agonist evoked the half-maximal peak calcium amplitude response; this dose was used for subsequent modulation experiments. SCN neurons showed reproducible calcium rises to glutamate and kainate. The \( EC_{50} \) for glutamate was 2 \( \mu M \) \((n = 189) \) and for kainate was 50 \( \mu M \) \((n = 160) \). t-ACPD did not evoke calcium rises at doses up to 300 \( \mu M \) \((n = 161) \).

NMDA DOSE-RESPONSE CURVES. SCN neurons showed reproducible calcium responses to NMDA. Increasing concentrations of NMDA were applied twice for 15 s each in Mg\(^{2+}\)-free buffer. Results were analyzed as described previously for kainate. The \( EC_{50} \) for NMDA-evoked calcium rises was 10 \( \mu M \) \((n = 74) \) (Fig. 3A). NMDA responses could be completely inhibited by 2 mM Mg\(^{2+}\), the concentration in the standard

**FIG. 1.** Punch explant cultures contain neurons and glia. A: MAP2 staining of suprachiasmatic nucleus (SCN) punch explant. B: GFAP staining of SCN punch explant. C: fura-2 loaded cells as seen during a typical experiment; neurons are identifiable as bright cells with compact cell bodies and respond to N-methyl-d-aspartate (NMDA).
perfusion buffer. To determine the dose at which the calcium response to 10 mM NMDA could be blocked by only 50%, Mg^2+ dose-response experiments were performed (n = 245) (Fig. 3B). Subsequent modulation experiments involving NMDA were performed with buffer containing this EC_{50} concentration of Mg^2+ (250 μM) and 2 μM glycine.

**FIG. 3.** Glutamate receptor agonist dose-response curves in SCN neurons. SCN punches were obtained from P1 rats, mildly dispersed, and plated on glass coverslips. Cells were loaded with fura-2, and neurons were identified as compact phase-bright cells. Shown are dose-response curves for SCN neurons for glutamate (●), kainate (▲), and trans-(±)-1-amino-1,3-cyclopentanedicarboxylate (t-ACPD) (■). The peak responses to each dose (applied in duplicate) were plotted as a percentage of the peak response to the maximal dose. The EC_{50} for glutamate was 3 μM (n = 189) and for kainate was 50 μM (n = 160). t-ACPD did not evoke repeatable calcium rises at doses up to 300 μM (n = 161).

Modulation of iGluR responses by mGluRs

SCN neurons show mGluR inhibition of iGluR-evoked calcium rises much like those reported for SCN astrocytes. Type I/II mGluR agonists t-ACPD (100 μM) and L-CCG-I (10 μM) were applied for 2 min before and during a 15-s kainate (50 μM) pulse. Although not evoking calcium rises, both mGluR inhibition of iGluR-evoked calcium rises much like those reported for SCN astrocytes. Type I/II mGluR agonists t-ACPD (100 μM) and L-CCG-I (10 μM) were applied for 2 min before and during a 15-s kainate (50 μM) pulse. Although not evoking calcium rises, both mGluR agonists inhibited calcium rises evoked by kainate (50 μM) in a dose-dependent manner (Fig. 4). The % decrease of kainate-evoked calcium rises was calculated as follows: difference in amplitude normalized by dividing by the kainate-only amplitude and then multiplied by 100 to obtain a percentage change. The minimum cutoff used to indicate a modulatory interaction was a 15% change in calcium rise amplitude. Negative numbers indicate an inhibition of the kainate calcium rise.

**FIG. 4.** L-CCG-I inhibition of iGluR-mediated Ca^{2+} rises. A: (2S,1′S,2′S)-2-(carboxycyclopropyl)-glycine (L-CCG-I) (10 μM) inhibited calcium rises evoked by kainate (50 μM). B: both 10 μM L-CCG-I (n = 30/72) and 100 μM t-ACPD (n = 30/114) inhibited kainate-evoked calcium rises to a similar degree. To determine percentage change of kainate-evoked calcium rises, the amplitude of kainate-evoked calcium rises was subtracted from the calcium rise evoked by kainate plus the mGluR agonist. Difference in amplitude was normalized by dividing by the kainate-only amplitude and then multiplied by 100 to obtain a percentage change. The minimum cutoff used to indicate a modulatory interaction was a 15% change in calcium rise amplitude. Negative numbers indicate an inhibition of the kainate calcium rise. C: calcium rises evoked by NMDA (10 μM) also were inhibited by 10 μM L-CCG-I (26.2 ± 2.6%, n = 13/28). This representative neuron also shows inhibition of NMDA-evoked calcium rises by the L-type calcium channel blocker nimodipine (NIM). Most SCN neurons tested showed >15% inhibition by 1 μM nimodipine (~44.4 ± 2.7%, n = 44/46).
agonists inhibited kainate-evoked calcium rises to a similar degree: t-ACPD, −42.2 ± 2.1%, n = 30/114; L-CCG-I, −30.9 ± 2.2%, n = 30/72 (Fig. 4B). Similar experiments showed that the mGluR agonist L-CCG-I inhibited NMDA-evoked calcium rises in SCN neurons (Fig. 4C); 10 μM L-CCG-I inhibited the NMDA calcium rise by −26.2 ± 2.6% (n = 13/28).

**Signal transduction pathways**

**G proteins.** Inhibition of kainate-evoked calcium rises by L-CCG-I does not appear to involve a Gi family G protein; the interaction is not blocked by overnight treatment with 200 ng/l PTx. L-CCG-I (10 μM) inhibits kainate (50 μM) evoked calcium rises by −32.9 ± 2.5% (n = 29/77) in PTx-treated SCN neurons, similar to cultures not treated with PTx. This suggests that mGluR modulation of kainate responses may involve a PTx-insensitive G protein such as Gq.

**PKC.** Some mGluRs are linked to activation of PKC. In addition, PKC has been implicated in modulation of circadian clock light responses. To investigate whether PKC is involved in mGluR inhibition of iGluR-evoked calcium rises, whether PKC activators could mimic mGluR inhibition and then whether PKC inhibitors could block mGluR effects were tested.

Calcium rises evoked by a 20-s application of 100 μM glutamate were similarly inhibited by the PKC activator PMA in SCN astrocyte cultures (n = 12) and in astrocytes and neurons in SCN punch explants (n = 31) (Fig. 5A); 500 nM PMA was applied for 2–3 min before the glutamate pulse. Similarly, PMA reduced kainate-evoked rises in SCN neurons by 48.8 ± 3.2 nM (n = 31/37) (Fig. 5B). PKC activation by 200 μM PDBu (−42.4 ± 2.6%, n = 46) or 60 μM OAG (−48.9 ± 1.9%, n = 59) also inhibited kainate-evoked calcium rises.

In the presence of PMA (500 nM), the inhibitory effects of t-ACPD on kainate-evoked calcium rises were larger (t-ACPD alone: −49.3 ± 4.8%; t-ACPD + PMA: −81.4 ± 4.1%; P = 0.0001, n = 23) (Fig. 5B). PDBu, a phorbol ester that washes out more easily than PMA, showed similar effects. t-ACPD inhibited the kainate-evoked calcium rise by −53.5 ± 1.6% (n = 57). After 15-min incubation with PDBu (200 μM), t-ACPD inhibited the kainate calcium rise by −82.8 ± 1.2% (P < 0.0001) in the same neurons. The PKC inhibitor H7 (200 μM) reversed the inhibitory effects of mGluRs (t-ACPD alone: −40.1 ± 2.8, t-ACPD + H7: 103.8 ± 33.1; P = 0.0022, n = 10/46) (Fig. 5B).

**OTHER SECOND MESSAGERS.** Activation of type II mGluRs may inhibit adenylyl cyclase and thereby reduce cAMP-dependent PKA levels. Other transmitters linked to PKA modulate light and glutamate responses of SCN cells. To test whether changing cAMP levels could modulate inhibitory effects of mGluRs, cells were treated with the cAMP analog 8Br-cAMP (1 mM). Cells were tested three or four times with 20-s applications of kainate, incubated with t-ACPD (100 μM) or L-CCG-I (10 μM) for 2–3 min before and during kainate pulses, washed, treated with kainate, treated with 8Br-cAMP for 1 min before the mGluR agonist incubation, and tested with kainate again. This was repeated in three separate SCN explants. t-ACPD inhibition was significantly attenuated in the presence of 8Br-cAMP (ACPD alone: −31.8 ± 2.6%, ACPD + 8Br-cAMP: −20.4% ± 4.3%; n = 32/73; P = 0.0290) (Fig. 5B). 8Br-cAMP had similar effects on L-CCG-I inhibition of kainate-evoked calcium rises (pre: −32.4 ± 1.3%, post: −23.7 ± 2.0%; n = 47/62; P = 0.0005). These results indicate that mGluR inhibition of kainate calcium rises may be influenced by both PKC and PKA.

**VACCs**

iGluR-activated calcium rises mediated by VACCs. In SCN neurons, kainate and NMDA-evoked calcium rises involve calcium influx through L-type calcium channels (Fig. 6). Kainate calcium rises are reduced by the L-type calcium channel antagonist nimodipine (1 μM) in 84 of 93 cells (−50.2 ± 2.0%), but no cells showed >15% inhibition by the N-type calcium channel inhibitor ω-conotoxin (1 μM) (−8.7 ± 1.8%, n = 28). NMDA calcium rises were similarly affected by nimodipine (−44.4 ± 2.7%, n = 44/46). Calcium rises evoked by membrane depolarization with 20 mM K+ were primarily attributable to calcium influx through L-type channels, whereas blockade of N-type channels had no effect on rise amplitude.
although 1–3 min pretreatment with PKC activators was blocked by 1 mM 8Br-cAMP (13.0, 6
25/31) or calphostin C (250 nM, 2
L-CCG-I were blocked by 1 mM 8Br-cAMP (13.0, 6
1
K
PKC activators H7 (200 µM, −47.5 ± 4.4%, n = 25/31) or calphostin C (250 nM, −48.5 ± 5.7%, n = 21/23) (Fig. 7C). These data suggest that the L-CCG-I inhibition of K
1-evoked Ca
2
rises was not due to PKC activation.

Unlike PKC activators, the PKA activator 8Br-cAMP (1 mM) did not significantly change K
1-evoked calcium rises (−13.3 ± 2.7%, n = 42/44). However, the inhibitory effects of L-CCG-I were blocked by 1 mM 8Br-cAMP (13.0 ± 6.0%; n = 42/44; vs. L-CCG-I alone, P < 0.0001) (Fig. 7D). This suggests that mGluR inhibition of VACCs is modulated and may even be directly mediated by PKA.

PKC–PKA interactions

PKC and PKA were both implicated in phase-dependent circadian clock resetting. Both are second messengers activated by mGluR signaling pathways. Could coincident activation of these two second messenger pathways modulate glutamate responsiveness? In SCN neurons, co-activation of PKC and type I/II mGluRs has synergistic effects on intracellular calcium (Fig. 8A). The nonphorbol ester PKC activator OAG (60 µM) did not itself induce a rise in intracellular calcium; however, when applied with L-CCG-I, a large calcium rise was rapidly evoked (211.4 ± 26.6 nM, n = 23/38). Interestingly, this calcium rise was reversibly blocked by addition of 20 mM K
1 to the perfusion medium.

Similarly, PKA activation by 8Br-cAMP (1 mM) had no effect on basal calcium levels. However, when co-applied with OAG (60 µM) an average 123.5 ± 13.6 nM increase in basal calcium concentration was produced, a 202.5 ± 18.5% change over basal levels (n = 50). As shown in Fig. 8B, these effects were reversible over time and were not affected by order of drug application.

Discussion

Evidence is provided that demonstrates that iGluR calcium responses in cultured SCN neurons are inhibited by activation of type II mGluRs. Also, it is shown that mGluR inhibition can be modulated or mediated by PKC and PKA. Inhibition of iGluR responses appears to occur at L-type calcium channels, although modulation of K
1 channels or iGluRs cannot be ruled out. Modulation may occur via mGluR-dependent or -independent activation of multiple second messenger pathways. Regulation of intracellular calcium levels may be an important means of modulating circadian clock responses to environmental stimuli.
Modulation of excitatory activity in SCN neurons

Throughout the CNS, activation of mGluRs may provide one of the primary means for reducing transmission at glutamatergic synapses (reviewed by Conn and Pin 1997). These findings suggest this is also true in the SCN; the type I/II mGluR agonists t-ACPD and L-CCG-I decrease kainate- and NMDA-evoked calcium rises in SCN neurons. Interestingly, the inhibitory effects of mGluRs were not blocked by activation of PKC, as was found in corticostriatum and hippocampus (Macek et al. 1998; Swartz et al. 1993). In contrast, PKC agonists mimicked and exacerbated the inhibitory effects of mGluRs on iGluR-evoked calcium rises. PKC effects were reversed by the PKC inhibitor H7. When co-applied with mGluR agonists, PKC activators increased mGluR inhibition. Taken together, these findings suggest PKC effects are at least partly due to a mGluR-independent pathway. How might these mGluR dependent and independent pathways converge to modulate glutamatergic transmission?

1) mGluRs may be directly activating PKC or PKA pathways. In some systems, mGluR activation inhibits adenylate cyclase, leading to decreased cAMP levels (Genazzini et al. 1993; Tanabe et al. 1993). mGluRs are also linked to PKC activation (Angenstein et al. 1997; Fukunaga et al. 1992; Harvey and Collingridge 1993).

These results suggest that t-ACPD and L-CCG-I are acting on a type II mGluR in SCN neurons. t-ACPD and L-CCG-I have micromolar affinities for both type I and type II mGluRs (Hayashi et al. 1993; Pin and Duvoisin 1995). Type I mGluRs are commonly linked to phosphoinositol hydrolysis and consequent increases in intracellular calcium (Abe et al. 1992). Type II mGluRs are coupled to decreases in cAMP and inhibition of adenylate cyclase (Tanabe et al. 1993). At the doses used, neither t-ACPD nor L-CCG-I evoked repeatable calcium rises in cultured SCN neurons. Because of this it is likely that the modulation seen is attributable to a type II mGluR. In addition, mGluR activation did appear to reduce cAMP levels in SCN neurons. Increasing cAMP levels with 8Br-cAMP reversed the inhibitory effects of mGluRs on iGluR-evoked calcium rises; 8Br-cAMP had no effect on basal calcium levels. However, it should be noted that even within receptor classes mGluR subtypes may show differing agonist sensitivities (McCullough et al. 1996).

PKC, rather than being a downstream effector, appears to modulate mGluR effects. Unlike 8Br-cAMP, PKC activators mimicked the inhibitory effects of mGluRs on iGluR-evoked calcium rises. mGluR effects were reversed by the PKC inhibitor H7. When co-applied with mGluR agonists, PKC activators increased mGluR inhibition. Taken together, these findings suggest PKC effects are at least partly due to a mGluR-independent pathway. How might these mGluR dependent and independent pathways converge to modulate glutamatergic transmission?

2) mGluR-independent activation of PKC/PKA pathways may modulate mGluR effects. Interactions between input pathways or feedback onto the SCN by nonglutamatergic input pathways may be one means of regulating glutamate responsiveness. Several transmitters and modulators have been localized to the SCN (van den Pol and Tsujimoto 1985). Many of the receptors for these transmitters are coupled to second messenger pathways. PKC and cAMP are implicated in modulation of the effects of light and glutamate on the SCN (Hannibal et al. 1997; McArthur et al. 1997). In addition to NPY and melatonin, serotonin and dopamine have also been shown to modulate glutamate and light responses in the SCN (McNulty et al. 1998; Rea et al. 1994; Srkalovic et al. 1994; Viswanathan and Davis 1997).

Independent actions of PKC or PKA on mGluR targets may influence the effectiveness of mGluR agonists. For example, results shown here and by other researchers show that PKA and PKC can modulate VACCs and iGluRs by non-GluR pathways (McCullough and Westfall 1996; Raymond et al. 1993; Wang et al. 1993). Multiple effects of kinases may be one explanation for the different inhibition response rates seen between mGluR and PKC agonists in this study. Differential activation of convergent pathways may also regulate glutamate responsiveness. Other researchers have shown that mGluRs may be linked to multiple second messenger cascades via distinct G proteins, possibly dependent on intracellular calcium concentration (Aramori and Nakanishi 1992; Sahara and Westbrook 1993). In this paper, co-activation of PKC and PKA dramatically increased calcium levels, whereas activation of either substrate alone had no effect on basal calcium. It is tempting to speculate that coincident activation of PKC and PKA may regulate mGluR signaling in SCN cells.

Second messenger targets

These experiments strongly suggest that both cAMP and PKC influence mGluR modulation of excitatory activity in SCN neurons. What are the cellular targets of these second messengers? Activation of kainate receptors leads to mem-
brane depolarization and activation of VACCs. In SCN neurons, over one-half the calcium rise evoked by perfusion with kainate is blocked by the L-type VACC antagonist nimodipine. Could second messengers be modulating these calcium channels in SCN cells? In several systems, PKC activation inhibits L-type VACCs (Bourinet et al. 1994; Doerner et al. 1988; Haymes et al. 1992; Johnson et al. 1993). Similarly, calcium channels can be modulated by cAMP and PKA (Desruies et al. 1993; Frings et al. 1992; Goulding et al. 1992). Decreasing cAMP levels and inhibition of PKA may lead to dephosphorylation and inhibition of calcium channels (Pemberton and Jones 1997; Perez-Reyes et al. 1994; Yu et al. 1993). Cyclic nucleotide-gated channels are integral to phototransduction and may play a role in circadian clock regulation (Akasu and Shoji 1994; Nawy and Jahr 1993).

Thus it was investigated whether PKC and cAMP could modulate VACCs in SCN neurons. Interactions with calcium rises evoked by increasing extracellular K+ concentration were studied because more than one-half the amplitude of these evoked calcium rises is attributable to L-type VACCs. In most SCN neurons, both type I/II mGluR and PKC agonists reduced K+-evoked calcium rises. However, mGluR inhibition of VACCs was not affected by specific PKC inhibitors. Although these results indicate that type I/II mGluR inhibition of VACCs is not mediated by PKC, they also provide evidence that modulation of iGluR calcium rises may not entirely be due to effects on VACCs. PKC may be acting on a substrate different from VACCs, possibly the kainate channel itself. PKC has been shown to inhibit kainate channel activity by direct phosphorylation (Dildy-Mayfield and Harris 1994).

In contrast, mGluR inhibition of K+-evoked calcium rises was blocked by the cAMP analogue 8Br-cAMP. Together with the results showing that 8Br-cAMP reduced mGluR inhibition of iGluR calcium responses, this suggests that type II mGluR inhibition of iGluRs is mediated by cAMP.

mGluR activation may inhibit calcium entry through VACCs by altering membrane polarization, thereby preventing activation of these voltage-dependent channels. mGluR activation alters neuronal membrane properties by modulating K+ channel function in neurons from several brain areas (Saugstad et al. 1995). Stimulation of mGluRs in some systems activates a G protein that has a direct effect on specific K+ channels (Sharon et al. 1997). This may explain the finding in SCN neurons that K+ blocked the calcium increase evoked by the coactivation of PKC and mGluRs.

mGluR activation may alter network properties in SCN explant cultures. Several researchers have shown active synapses in cultured hypothalamic cells (Obristian and van den Pol 1995; Welsh et al. 1995). Decreased glutamate release mediated by mGluRs could decrease excitability and lead to apparent inhibition of VACCs. Another explanation for the inhibitory effects of t-ACPD is the modulation of GABA release. GABA is the primary neurotransmitter in the SCN (van den Pol and Tsujimoto 1985). There is evidence that modulation of calcium channels, of which experiments presented here imply, are as one effect of mGluR activation, can depress GABA release in SCN cells (Chen and van den Pol 1998). In support of this hypothesis, t-ACPD effects on light-induced phase shifts (Haak et al. 1997b) mirror those of the GABA antagonist bicuculline (Gillespie et al. 1996, 1997).

Functional implications

mGluRs perform many modulatory functions in the nervous system, including regulation of fast synaptic transmission, changes in synaptic strength, and reduction of neurotoxicity. These effects often appear to be mediated by inhibition of presynaptic VACCs and consequent reduction in transmitter release (Lovingier 1991; Manzoni and Bockaert 1995; Taka-hashi et al. 1996; Yoshino and Kamiyama 1995).

Scott and Rusak (1996) showed that t-ACPD modulated spontaneous neural activity in SCN slices preferentially in the ventrolateral retinorecipient area. Results presented in this study suggest that mGluRs inhibit excitatory transmission in SCN neurons. VACCs, modulatable by PKC and PKA, appear to be one component of this inhibition. Results from other support a role for calcium channels in clock timing. Benloucif et al. (1997) provide preliminary evidence that nimodipine enhances light-induced phase delays at CT14. In addition, Ding et al. (1998) suggest that intracellular calcium release mediates circadian clock resetting only during the early night. Coupled with results presented here, these findings indicate that mGluRs may play a significant role in modulating photic transduction in the SCN.

L. L. Haak was supported by National Institute of Mental Health Training Grant MH-17047-15.

This research was funded by the Air Force Office of Scientific Research. L. L. Haak thanks Drs. H. E. Albers, H. C. Heller, E. M. Mintz, and A. N. van den Pol for guidance, support, and helpful discussions and Drs. James T. Russell and Kenneth Maiese for comments on the manuscript.

Present address and address for reprint requests: Laboratory For Cellular and Molecular Neurophysiology, NIH, Bldg. 40, Rm. 5C-28, Bethesda, MD 20892-4495.

Received 17 August 1998; accepted in final form 9 November 1998.

REFERENCES


PEMBERTON, K. E. AND JONES, S.V.P. Inhibition of the L-type calcium channel by the five muscarinic receptors (m1–m5) expressed in NIH 3T3 cells. Pfaffner Arch. 433: 505–514, 1997.


RUPER, J. AND SCHLOSSER, M. C. Calcium influx and modulation of recombinant GluR6 glutamate receptors by 


