GABA_B presynaptically modulates suprachiasmatic input to hypothalamic paraventricular magnocellular neurons

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Cui, Lu-Ning, Elaine Coderre, and Leo P. Renaud. GABA_B presynaptically modulates suprachiasmatic input to hypothalamic paraventricular magnocellular neurons. Am J Physiol Regulatory Integrative Comp Physiol 278: R1210–R1216, 2000.—This study used whole cell patch clamp recordings in rat hypothalamic slice preparations to evaluate the effects of GABA_B receptor activation on GABA_A-mediated inhibitory postsynaptic currents (IPSCs) in paraventricular nucleus magnocellular neurons evoked by electrical stimulation in the suprachiasmatic nucleus (SCN). Baclofen induced a dose-dependent (1–10 µM) and reversible reduction in SCN-evoked IPSC amplitude (11/11 cells), blockable with 2-hydroxyacldofen (300 µM; 3/3 cells). IPSCs displayed paired-pulse depression (PPD), attenuated by both baclofen and 2-hydroxyacldofen, but neither altered resting membrane conductances or IPSC time constants of decay. Baclofen induced a significant dose-dependent (1–100 µM) reduction in frequency, but not amplitude, of spontaneous IPSCs and miniature IPSCs, reversible with 2-hydroxyacldofen pretreatment. Baclofen effects and PPD persisted in slices pretreated with pertussis toxin (PTX) and N-ethylmaleimide, implying that these GABA_B receptors are coupled to PTX-insensitive G proteins. Responses were unaltered by barium (2 mM) or nimodipine, ruling out involvement of K^+ channels and L-type Ca^2+ channels. Thus pre- and postsynaptic GABA_B receptors and GABA_A receptors participate in SCN entrainment of paraventricular neurosecretory neurons.

Electrophysiology; patch clamp recordings; circadian rhythms; oxytocin; vasopressin

In mammals, circulating levels of the neurohypophyseal hormones arginine vasopressin (AVP) and oxytocin (Oxy) are regulated by the coordinated activity of magnocellular neurons in the hypothalamic paraventricular (PVN) and supraoptic nuclei (SON) and their accessory nuclei. Diurnal variations in plasma AVP and Oxy levels have been noted in both humans (21) and rats (11, 38). This could imply entrainment of these neurosecretory neurons by the hypothalamic suprachiasmatic nucleus (SCN), the site of a major biological clock regulating circadian rhythms in the mammalian brain (19). Given the importance of AVP and Oxy in hydromineral homeostasis, there is a need to delineate the neural mechanisms involved in transferring SCN’s imprint on neuroendocrine function and hormone release in the neurohypophysis.

The PVN, widely regarded as a key brain center for control of autonomic and neuroendocrine function (29), would be a suitable target for entrainment by SCN. In fact, SCN does innervate the hypothalamic area around PVN (3, 35, 36). Additionally, consistent with the immunocytochemistry that reveals GABA in the somata of most SCN neurons (24) is the localization of GABA in axon terminals of these SCN projections (4). Interestingly, although anatomic data suggest that PVN is not a major target of SCN efferents (3, 4, 35, 36), electrophysiological data indicate that at least a portion of these efferents project directly to putative AVP- and Oxy-synthesizing magnocellular neurons to reduce their excitability via monosynaptic inputs that act via postsynaptic GABA_A receptors (13, 14). Therefore, rapid neurotransmission between SCN and PVN magnocellular neurons appears to be mediated via ionotropic GABA receptors, consistent with immunocytochemical and functional impressions.

An alternate role for GABA acting at metabotropic GABA_B receptors may be anticipated given the recent evidence of expression of GABA_B receptors in hypothalamus (17) and evidence that they may contribute to modulation of synaptic inputs intrinsically within SCN itself (5) or to magnocellular neurons in the SON (18, 20, 23). To address whether GABA_A receptors might have a role in modulating a “circadian-relevant” pathway to the neuroendocrine hypothalamus, we investigated the effects of GABA_A receptor agonists and antagonists on fast inhibitory neurotransmission evoked in magnocellular PVN neurons by SCN stimulation. We conclude that this innervation, tentatively involving both AVP- and Oxy-synthesizing PVN neurons, is subject to inhibitory modulation by presynaptic GABA_B receptors.

Materials and Methods
Preparation. Experiments used brain slices prepared from Long-Evans rats (100–250 g) housed under a 12:12-h light-dark regimen for at least 2 wk. Under methoxyflurane (Janssen) anesthesia, rats were decapitated and their brains were rapidly removed and placed in ice-cold oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (aCSF) composed of (in mM) 124 NaCl, 3.2 KCl, 26.2 NaHCO_3, 1.3 MgCl_2, 1 NaH_2PO_4, 10 glucose, and 2.4 CaCl_2 at pH of 7.4. Coronal slices 500 µm in thickness containing both PVN and SCN were cut with a vibratome, trimmed, and preincubated in...
aCSF for ≥1 h at room temperature and transferred to a submerged chamber for recordings at room temperature.

Electrophysiology. A concentric bipolar stimulating electrode (OD 325 µm, tip-ring separation 100 µm; FHC, Bowdoinham, ME) positioned in SCN and connected to a stimulus isolation unit was used to evoke postsynaptic events in PVN neurons. Recordings were obtained during the subjective dark cycle, using the whole cell patch clamp technique with micropipettes (borosilicate glass: OD 1.5 mm; ID 1.1 mm) filled with a solution containing (in mM) 90 K gluconate, 50 KCl, 10 HEPES, 1 EGTA, 2 Mg-ATP, and 0.2 GTP at pH 7.3 at an osmolarity of 285 mosM; biocytin 5 mM was added for cell visualization after intracellular dialysis. A higher chloride concentration was used to amplify inhibitory postsynaptic currents (IPSCs) at a holding potential near membrane resting potential. Pipettes had an open resistance of 3–7 MΩ and seal resistances were ≥3 GΩ. Values were not corrected for liquid junction potential. Data were obtained with an Axopatch 1-D amplifier (Axon Instruments) and low-pass filtered at 2 kHz. Clampex 6.0 or 7.0 (Axon Instruments) was used for online data acquisition, and stored data were analyzed using Clampfit 6 (Axon Instrument) and Mini Analysis Program (version 3.0, Synaptasoft, Leonia, NJ). Results are expressed as means ± SE.

Histological identification. Slices were fixed in 4% paraformaldehyde in 0.1 M PBS overnight and subsequently immersed in PBS containing 20% sucrose (wt/vol), then sectioned on a cryostat (40 µm), and stained with streptavidin-conjugated Texas red. Although an earlier study (13) indicated that SCN stimulation evoked monosynaptic responses in PVN, these cells displayed properties attributed to oxytocin-synthesizing neurons. In experiments to test for clamping or, alternatively, slices were preperfused with N-ethylmaleimide ( Sigma, St Louis, MO) 50 µM for 5 min before application of the GABA<sub>B</sub> receptor agonist.

RESULTS

Identification of magnocellular cells and SCN-evoked IPSCs. Recordings were restricted to neurons anatomically localized within the magnocellular part of the PVN. These cells displayed properties attributed to magnocellular neurons: mean membrane potential of −54.5 ± 0.8 mV, membrane resistance of 864.2 ± 51.4 MΩ, linear current-voltage plots at resting potential levels (Fig. 1Aa), frequency-dependent action potential broadening (Fig. 1Ab), expression of a transient outward current ("A" notch) when a depolarizing current injection is initiated from a hyperpolarized resting membrane potential (Fig. 1Ac; see Refs. 14, 32). By analogy with observations in SON, where immunocytochemically characterized AVP- and Oxy-synthesizing neurons demonstrated differences in their response to hyperpolarizing current pulses triggered from "depolarized" resting levels of −50 mV (28), we tentatively classified (without immunocytochemical confirmation) 22 neurons as putative Oxy-synthesizing cells on the basis of their demonstration of a time-dependent inward rectification to an inward current pulse triggered from a holding level approximately −50 mV and a rebound depolarization of varying magnitude upon return to resting membrane potentials (Fig. 1Ba). Another 20 neurons with little or no time-dependent rectification and delayed return to baseline from hyperpolarized levels (Fig. 1Bb) were tentatively labeled as

![Fig. 1. A: current clamp traces illustrate electrophysiological properties attributed to magnocellular neurosecretory neurons. Aa: response to a series of intracellular current pulses; Ab: broadened action potential; Ac: delayed onset of action potential firing when the membrane potential is stepped from a hyperpolarized level (−82 mV) to a depolarized level. B: when held at a potential of −50 mV, responses to a hyperpolarizing current pulse reveal 2 patterns. Ba: bottom trace from cell illustrated in Aa displays time-dependent rectification and a small rebound depolarization (arrow), tentatively attributed to oxytocin-synthesizing neurons. Bb: trace from a cell that lacks time-dependent rectification and displays a delayed return to baseline (open arrow), tentatively identified as a vasopressin-synthesizing neuron. C: control traces (average of 5 sweeps) obtained in voltage clamp illustrate suprachiasmatic nucleus (SCN)-evoked inhibitory postsynaptic currents (arrow designated by *), their blockade in media containing 20 µM bicuculline, and recovery 15 min after drug washout.]
AVP-synthesizing cells. Consistent with earlier observations in this preparation (13), SCN stimulation evoked IPSCs, blockable with bicuculline (Fig. 1C); their constant latencies and ability to follow stimuli >20 Hz implied a monosynaptic connection.

Effects of baclofen and 2-hydroxysaclofen on SCN-evoked IPSCs. In the presence of a GABA B receptor agonist, baclofen (1–10 µM), all of 11 cells tested (holding potential = −70 mV; 6 putative Oxy- and 5 putative AVP-synthesizing cells) demonstrated a dose-dependent and reversible reduction in the amplitude of SCN-evoked IPSCs (Fig. 2, A–C). Despite the amplitude reduction, time constants of decay of SCN-evoked IPSCs were not altered significantly (22.2 ± 1.3 ms in control, 22.9 ± 4.7 ms) in the presence of baclofen (n = 7, P > 0.5, paired Student’s t-test). In three of three cells tested, the baclofen-induced effects could be prevented by prior perfusion with aCSF containing 2-hydroxysaclofen (300 µM). The observation that the amplitude of SCN-evoked IPSCs did not change in the presence of 2-hydroxysaclofen suggests little or no tonic activation of GABAB receptors by endogenously released GABA at this particular synapse.

Baclofen and 2-hydroxysaclofen alter paired pulse depression by different mechanisms. Paired-pulse paradigms can be useful in the identification of presynaptic mechanisms (e.g., Refs. 7, 22). All of 12 cells tested with paired SCN stimuli revealed a reduction in the amplitude of the second response (P2) over interstimulus intervals between 100 and 700 ms, peaking around 150–200 ms, i.e., paired-pulse depression or PPD (Fig. 3). Both baclofen and 2-hydroxysaclofen attenuated PPD, although in a different manner. In the presence of 3 µM baclofen, there was a reduction in the amplitude of both evoked IPSCs, similar to the actions reported above. However, in baclofen the P2/P1 amplitude ratio...
also changed from 0.64 ± 0.10 (control) to 1.06 ± 0.28 (n = 7 cells; P < 0.05; Fig. 3, A and B), in effect abolishing PPD through reduction in the amplitude of the P1 response. In the presence of 300 µM 2-hydroxyasaclofen, which did not alter the amplitude of P1 response, the P2/P1 ratio of the evoked IPSCs also changed from 0.56 ± 0.05 (control) to 0.89 ± 0.12 (n = 5 cells; Fig. 3, C and D). Although this also produces reduction in PPD (note the resemblance between histograms in Fig. 3, D and B), the mechanism is different because of an increase in the amplitude of the P2 response. Neither baclofen nor 2-hydroxyasaclofen significantly altered amplitudes of P1 or P2 responses. Baclofen action was a baclofen-induced, dose-dependent, and reversible reduction in the frequency of spontaneous IPSCs (sIPSCs; n = 6 cells; Fig. 4, A and C). Tests on three cells indicated that this effect could be prevented by prior addition of 2-hydroxyasaclofen (300 µM; Fig. 4A). Cumulative probability plots obtained from recordings in the presence of 1 and 10 µM baclofen (Fig. 4B) indicated no significant change in sIPSC amplitude but a marked reduction in the frequency of occurrence of sIPSC frequencies. In media containing 0.5 µM tetrodotoxin, baclofen had similar depressant actions on the frequency of occurrence, but not amplitude, of miniature IPSCs (mIPSCs; n = 4 cells; not illustrated). Interestingly, in tests on six cells with ACSF containing 300 µM 2-hydroxyasaclofen, all displayed a significant increase in sIPSC frequency (from 1.13 ± 0.16 to 3.03 ± 0.61 events/s) but not amplitude (44 ± 9.0 vs. 48 ± 7.7 pA).

PTX-sensitive G protein involvement. At the neuronal somata, GABA_B receptors are known to activate G proteins, and their actions are mediated through altered conductances involving inwardly rectifying K^+ channels or suppression of Ca^{2+} channels (1, 9). However, there is some question as to the channels that mediate responses to activated presynaptic GABA_B receptors and whether these also involve G proteins (33). To test whether responses to baclofen might use PTX-sensitive G proteins, recordings were made from slices that had been incubated in ACSF containing PTX (1 µg/ml) for 16–20 h. Data from paired SCN-evoked IPSCs in three neurons in different slices revealed a P2/P1 ratio of 0.58 ± 0.05, indicating persisting PPD. In the presence of baclofen, the amplitude of SCN-evoked IPSCs was reduced by 45.9 ± 8.9%, altering the amplitude of the P1 response and changing the PPD ratio to 0.85 ± 0.05. In tests on two other neurons in control slices, preperfusion with N-ethylmaleimide, a sulfhydryl-alkylating agent that has been shown to block G protein-effector interactions by alkylating the α-subunits of PTX-sensitive GTP-binding proteins (26, 34), did not change the ratio of PPD nor the response to baclofen (not illustrated).

Potassium vs. calcium channel involvement in baclofen's effects. Ionic mechanisms mediating responses to these presynaptic GABA_B receptors were examined briefly by perfusion of slices with different ionic channel blockers. In the presence of 2 mM Ba^{2+}, a nonselective K^+ channel blocker, the depressant effects of

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Fig. 4. A: current traces from a single paraventricular nucleus (PVN) neuron. Control traces reveal abundant spontaneous IPSCs (sIPSCs). Top right, note reduced frequency of sIPSCs in 10 µM Bac. Bottom left, traces reflect blockade of Bac effect by prior and simultaneous application of 300 µM Sac. Bottom right, eventual return of Bac effect after Sac washout. B: results of cumulative probability analyses of amplitude and interevent intervals of spontaneous IPSCs in control (solid line) and during exposure to 10 µM Bac (dashed line). Note shift to right in interevent intervals but no change in the amplitude of sIPSCs. C: summary plot for spontaneous IPSCs observed in individual cells tested with different concentrations of Bac (open symbols), and mean values for these data (closed symbols). Although ANOVA test did not show a significant difference (P = 0.15 for frequencies, and P = 0.91 for amplitude), results reflect trend of a dose-dependent Bac-induced depression in frequency of occurrence of sIPSCs/s with little effect on their amplitudes.
badlofen on SCN-evoked IPSCs persisted and PPD was still evident in all of three cells tested, indicating that K channels may not be involved. Among the various types of calcium channels, the observation that nimodipine (10 µM) failed to alter badlofen’s action on SCN-evoked IPSCs or PPD (n = 3; data not illustrated) suggests lack of mediation via L-type channels. Tests with other blockers were not conclusive: application of both ω-agatoxin TK, a P/Q-type Ca\(^{2+}\) channel blocker (0.5 µM) in four cells and ω-conotoxin GVIA, an N-type Ca\(^{2+}\) channel blocker (2 µM) in three cells almost completely abolished SCN-evoked IPSCs. Other approaches will be required to assess whether P/Q- or N-type calcium channels mediate presynaptic GABA\(_B\) actions at this synapse.

**DISCUSSION**

In mammalian brain, the SCN is recognized as the locus of the main biological clock governing a majority of circadian rhythms (19). Because the PVN is regarded as a key center for the regulation of autonomic, neuro-endocrine, and cardiovascular function (29), the aim of this study was to provide further insight into the synaptic mechanisms through which SCN might entrain magnocellular neuroendocrine neurons. The electrophysiological data confirm the impression from immunocytochemical and functional studies that GABA is a principal neurotransmitter in SCN efferent pathways and that GABA acts at ionotropic postsynaptic GABA\(_A\) receptors to mediate rapid inhibitory neurotransmission from SCN to magnocellular neurons both in PVN (13) and SON (6). Consistent with the interpretation from earlier recordings in vivo (6, 14) and by analogy with the cell classification characteristics noted among immunocytochemically identified AVP- and Oxy-synthesizing cells in SON (28), we tentatively suggest that this innervation involves both AVP- and Oxy-synthesizing neurons.

In addition to its postsynaptic role in this specific pathway, we now propose that GABA also acts at presynaptic GABA\(_B\) receptors to modulate the inhibitory input from SCN to magnocellular neurons. Localization of the GABA\(_B\) receptors on the terminals of SCN afferents, rather than on the postsynaptic neurons, is supported by observations that badlofen, while markedly altering SCN-evoked IPSC amplitudes and reversing PPD, had no significant effect on either the time course of decay of SCN-evoked IPSCs or resting membrane currents and conductances (see Refs. 18, 20, 23 for SON neurons). A significant change in frequency, but not amplitude, of sIPSCs and mIPSCs in the presence of badlofen and 2-hydroxybadlofen is another reflection of a presynaptic GABA\(_B\) receptor-mediated action on GABA\(_B\)-mediated inputs to magnocellular neurons, albeit the origins of these sIPSCs cannot be defined. In each instance, receptor specificity is indicated by the blocking action of 2-hydroxybadlofen. The observation that the frequency of occurrence of sIPSCs was increased in the presence of 2-hydroxybadlofen suggests tonic activation of GABA\(_B\) receptors on magnocellular neurons. However, one might conclude that this was not the case for those synapses involved in SCN-mediated inputs because the 2-hydroxybadlofen treatment had little appreciable influence on the amplitude of the SCN-evoked compound IPSC. Alternatively, uncertainty on this issue arises because the stimulus intensities could have been supramaximal, thereby leaving little room for an amplitude increase. The fact that 2-hydroxybadlofen has little effect on the amplitude of the first of two SCN-evoked IPSCs but markedly enhances the amplitude of the second response suggests that PPD is due to endogenous action of GABA at the GABA\(_B\) receptor in the presynaptic terminals. Endogenous presynaptic activation of GABA\(_B\) receptors is also supported by the finding that 2-hydroxybadlofen increases the frequencies of spontaneous and miniature IPSCs without changing their amplitudes.

There is considerable information on the nature of the coupling of postsynaptic GABA\(_B\) receptors with G proteins and their effector ion channels. Much less is known about their presynaptic receptors and whether these may be a different subtype of GABA\(_B\) receptors as suggested by recent observations (e.g., Ref. 39). Given the evidence for the presynaptic location of the GABA\(_B\) receptors under study in this report, their resistance to pretreatment with agents that alter PTX-sensitive G proteins indicates the likelihood that these GABA\(_B\) receptors couple to ion channels via a PTX-insensitive G protein. Although our study was not definitive, the lack of effect of barium suggests mediation through Ca\(^{2+}\) rather than K\(^+\) channels. Similar conclusions have been reached in other central nervous system sites, including the calyces of Held in the medial nucleus of the trapezoid body (30) and hippocampal CA1 neurons (10). In the latter study, it was concluded that the presynaptic action of badlofen was mediated through both ω-conotoxin GVIA-sensitive and ω-agatoxin IVA-sensitive, but not dihydropyridine-sensitive, calcium channels. Although our preliminary data on this issue are inconclusive, they appear to eliminate involvement of L-type Ca\(^{2+}\) channels. It is interesting to note that the situation may vary with the area under study; recent studies (27) reveal that GABA\(_B\) receptors facilitate L-type and inhibit N-type calcium channels in salamander retinal neurons.

The presence of presynaptic GABA\(_B\) metabotropic receptors suggests that information transfer may not be linearly related to firing frequency of SCN neurons. Increased firing of SCN neurons, as noted during subjective light periods (15, 37) should lead to greater release of GABA. This would have the potential for competing consequences at the level of PVN magnocellular neurons: membrane hyperpolarization resulting from activation of postsynaptic GABA\(_B\) receptors, yet reduction in the effectiveness of postsynaptic inhibition due to the activation of presynaptic GABA\(_B\) receptors. This will be a matter for further investigations.

Although we and others (18, 20, 23) have seen no influence of badlofen on resting membrane currents and/or conductances in magnocellular cells, results from a recent study (12) indicate that magnocellular neurons in SON do have postsynaptic GABA\(_B\) recep-
GABA<sub>B</sub> receptors modulate SCN input to PVN

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Perspectives

Although circadian variations in plasma Oxy and vasopressin levels have been documented for sometime (11, 21, 38), mechanisms whereby the SCN imparts a circadian rhythmicity on the neurons synthesizing AVP and Oxy in the PVN and SON have remained undefined. Adding to the data available from immunocytochemistry and functional studies of SCN connectivity to a major hypothalamic center for neuroendocrine and autonomic function, the present observations support the importance of GABA in neuronal communication from SCN to magnocellular neurons. GABA mediates rapid neurotransmission from SCN to magnocellular neurons in PVN and SON via postsynaptic GABA<sub>B</sub> receptors (see Refs. 6, 13, 14). The present study indicates that in PVN, presynaptic GABA<sub>B</sub> receptors modulate this particular input. When viewed in a more global context, GABA<sub>B</sub> receptors can be seen to contribute to SCN function at several levels: modulation of excitatory retinohypothalamic inputs to SCN (16); modulation of activity within SCN, both at the cellular level (5) and globally, to induce phase shifts (2); and modulation of neurotransmission in SCN outputs, such as those to hypothalamic magnocellular neurons. Although GABA is a principal neurotransmitter in SCN neurons, future studies will need to account for several other coexisting peptides, some with demonstrable influences on circadian rhythms (31). Indeed there is complexity of function within this biological clock.

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