GABA\textsubscript{B} Receptor Modulation of Rapid Inhibitory and Excitatory Neurotransmission From Subfornical Organ and Other Afferents to Median Preoptic Nucleus Neurons

Miloslav Kolaj, Donglin Bai, and Leo P. Renaud. GABA\textsubscript{B} receptor modulation of rapid inhibitory and excitatory neurotransmission from subfornical organ and other afferents to median preoptic nucleus neurons. *J Neurophysiol* 92: 111–122, 2004. First published February 18, 2004; 10.1152/jn.00014.2004. Cardiovascular and behavioral responses to circulating angiotensin require intact connectivity along the upper lamina terminalis joining the subfornical organ (SFO) with the median preoptic nucleus (MnPO). Whole cell patch-clamp recordings in sagittal rat brain slice preparations revealed that 28/40 MnPO median preoptic nucleus (MnPO) neurons responded to electrical stimulation of SFO efferents with bicuculline-sensitive GABA\textsubscript{A} receptor-mediated inhibition and glutamate-mediated postsynaptic excitation involving AMPA and N-methyl-D-aspartate (NMDA) receptor subtypes, blockable with 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium (NBQX) and D-2-amino-4-phosphonovaleric acid (D-APV), respectively. Bath applications of baclofen induced a concentration-dependent (0.3–10 \( \mu \)M) reduction in these SFO-evoked postsynaptic currents, attenuation of SFO-evoked paired-pulse depression, and reduction in frequency (but not amplitude) of miniature postsynaptic currents, consistent with an action at presynaptic GABA\textsubscript{B} receptors. Baclofen’s effects on miniature currents lacked sensitivity to barium, \( \omega \)-conotoxin GVIA, and cadmium. Acting at postsynaptic GABA\textsubscript{B} receptors, baclofen hyperpolarized a majority of MnPO neurons by increasing a G protein–coupled inwardly rectifying potassium conductance and suppressing an N-type high-voltage–activated calcium conductance. The latter contributed to reduction in action potential afterhyperpolarization and enhanced cell firing and spike frequency adaptation when tested with a depolarizing stimulus. All baclofen-induced effects were blockable with CGP52432. CGP52432 alone had no significant effect on SFO-evoked postsynaptic current amplitudes or paired-pulse ratios, but did induce an increase in miniature inhibitory postsynaptic current (mIPSC) frequency in 2/4 cells tested, indicating that ambient levels of GABA could activate presynaptic GABA\textsubscript{B} receptors on undefined inputs. These observations indicate that MnPO neurons receive both a GABAergic and glutamatergic innervation from SFO. Both forms of rapid neurotransmission are subject to modulation via pre- and postsynaptic GABA\textsubscript{B} receptors.

**INTRODUCTION**

A variety of lesion and stimulation studies have indicated the importance of the lamina terminalis forming the anterior wall of the third cerebral ventricle in cardiovascular and hydromineral homeostasis (Johnson et al. 1996; McKinley et al. 1996, 1999). Three cell groups are located within this region. Two of these are circumventricular organs (CVOs), the organum vasculosum lamina terminalis (OVLT) situated ventrally and the subfornical organ (SFO) located dorsally, where fenestrated capillaries permit central neurons access to blood-born molecules (McKinley et al. 1991; Simon 2000). Information received by neurons residing in these CVOs is believed to be transmitted and integrated within the third cell group, the median preoptic nucleus (MnPO, also called MnPN, POMe, or nucleus medianus) located around the anterior commissure at the midpoint along the lamina terminalis. Indeed the connectivity of MnPO neurons suggests their suitability for reception and integration of messages derived from hemal and/or neural origins, based on reciprocal connections with neurons in SFO and OVLT (Miselis et al. 1979; Oldfield et al. 1992), afferent and efferent connections with neurons involved in hypothalamic neuroendocrine regulation and hydromineral balance (Armstrong et al. 1996; Oldfield et al. 1992; Silverman et al. 1981; Swanson 1976), sleep-waking cycles (Chou et al. 2002), and brain stem cardiovascular centers (Bester et al. 1997; Kawano and Masuko 1993; Lind and Swanson 1984; Saper and Levisohn 1983; Zardetto-Smith and Johnson 1995). Lesions centered on MnPO result in profound disruptions in cardiovascular regulation, fluid balance, angiotensin-induced drinking, and salt appetite (Cunningham et al. 1991; Gardiner and Stricker 1985; Gardiner et al. 1985, 1986; Mangiapane et al. 1983), attesting to the importance of this nucleus in maintaining homeostasis in autonomic, neuroendocrine, and cardiovascular systems.

It has long been known that the ability for circulating angiotensin to elicit a drinking response in the rat depends on the integrity of the SFO and its connectivity with MnPO (Eng and Miselis 1981; Lind and Johnson 1982). Whereas the intrinsic properties and neuropharmacology of SFO neurons have been revealed in some detail (reviewed in Washburn and Ferguson 2001), relatively little is known about the nature of the SFO projection to MnPO. In this study, we used patch-clamp recording techniques in brain slice preparations to characterize the responses elicited in MnPO neurons by electrical stimulation along the ventral edge of the SFO. Our observations indicate that both GABA\textsubscript{A} and glutamate ionotropic receptors mediate rapid neurotransmission from SFO to MnPO. Recent reports also identify MnPO with moderate to high levels of GABA\textsubscript{B}R1 immunoreactivity and mRNA (Bischoff et al. 1999; Margeta-Mitrovic et al. 1999), consistent with our data.
indicating the presence of both pre- and postsynaptic GABA\(_B\) receptors that may modulate SFO and other GABAergic and glutamatergic transmission to MnPO neurons.

**METHOIDS**

Experiments used male Long-Evans rats weighing 50–100 g. All experiments conformed to Canadian Council for Animal Care and Ottawa Health Research Institute guidelines on the ethical use of animals in research. Recordings were made from MnPO neurons in acutely prepared sagittal brain slice preparations, using methods described in detail previously (Bai and Renaud 1998a,b). Briefly, animals were decapitated, and the brain was quickly removed and immersed in a standard slicing solution (ACSF) of the following composition (in mM): 127 NaCl, 3.1 KCl, 1.3 MgCl\(_2\), 2.4 CaCl\(_2\), 26 NaHCO\(_3\), and 10 glucose, pH 7.3, osmolality 300–310 mOsm/kg. The brain was sliced in the sagittal plane with a vibratome, and a single midline slice 400–500 \(\mu\)m in thickness that contained the SFO and MnPO was preincubated in gassed ACSF for 1–2 h at room temperature and transferred to a superfused chamber superfused (2–3 ml/min) with oxygenated ACSF at 22–25 °C. Using the blind patch-clamp technique, recordings were obtained from MnPO neurons with borosilicate thin-walled micropipettes filled with (in mM) either 130 K-gluconate, 10 KCl, 10 NaCl, 2 MgCl\(_2\), 10 HEPES, 1 EGTA, 2 Mg-ATP, and 0.3 Na-GTP or cesium salts (e.g., Cs-methylsulfonate or CsCl) substituted for K-gluconate and KCl (pH adjusted with KOH or CsOH, respectively, to 7.3) when recordings required amplification of spontaneous and miniature inhibitory postsynaptic potentials (mIPSPs). Pipettes had resistances of 3–7 M\(\Omega\). Correction for liquid junction potential was applied to recorded membrane currents and voltages. Access resistance (<15 M\(\Omega\)) was considered acceptable. Input resistances were determined from the linear slope (i.e., between −50 and −80 mV) of the current-voltage \((I-V)\) relationships. Whole cell current-clamp and voltage-clamp recordings were obtained with an Axopatch 200B (Axon Instruments, Union City, CA). Data were filtered at 2 kHz, continuously monitored, and stored on videotape. Digidata 1200 interface and clampex (pClamp8) software (Axon Instruments) were used on-line to generate current and voltage commands.

Afferents to MnPO arising from the SFO were activated with a concentric bipolar electrode (25 \(\mu\)m tip diameter; FHC, Bowdoinham, ME) positioned at the ventral aspect of the SFO and connected to a stimulus isolation unit that delivered voltage pulses (1–30 V, 0.1 ms) under program control.

Off-line analyses were performed using Clampfit version 8 (Axon Instruments). Statistical comparisons between control and experimental values \((P < 0.05\) and better) were determined using both the paired or unpaired Student \(t\)-test and ANOVA. Results are expressed as means ± SE. For assessment of miniature postsynaptic events, 2–3 min of recordings (minimum of 100 events) were analyzed using Mini Analysis software from Synaptosoft (Leonia, NJ). Miniature events were defined as those recorded in the presence of 1 mM TTX and detected with an adjustable threshold that was maintained at a constant level in a given neuron. The analysis was performed by using cumulative probability plots, and statistical comparisons were evaluated with Kolmogorov-Smirnov (K-S) test.

Drugs were bath applied at the concentrations indicated. These included \((-\)\)-bicuculline methochloride (BIC), \((-\)\)-2-amino-5-phosphonovaleric acid (APV), 2,3-dioxo-fnitril-1,2,3,4-tetrahydrobenzo [\(f\)] quinoxaline-7-sulfoamide disodium (NBQX), \((R)-4\)-amino-3-(4-chlorophenyl) butanoic acid (Baclofen), guvacine, and CGP52432 from Tocris Cookson (Ballwin, MO), \(\alpha\)-conotoxin GVIA from Bachem (Torrance, CA), norepinephrine, Mg-ATP, Na-GTP, N0711, and cytochrome C from Sigma and/or Sigma-RBI (St. Louis, MO), and TTX from Alomone Laboratories (Jerusalem, Israel).

**RESULTS**

Recordings were obtained from MnPO neurons located both dorsal \((n = 24)\) and ventral \((n = 97)\) to the anterior commissure. Data samples obtained at the outset of whole cell recordings with K-gluconate-filled pipettes revealed a mean resting membrane potential of −55.1 ± 0.8 mV (range, −40 to −75 mV) and input conductance of 1.22 ± 0.1 nS. The fact that MnPO neurons display varying degrees of time-dependent or -independent inward rectification, and usually exhibit a low threshold spike on return of membrane potentials to rest from hyperpolarized levels (e.g., Fig. 1B, inset), suggests subpopulations of neurons. Spontaneous action potentials (range, 0.1–20 Hz) were noted in 85% of neurons; a minority had resting potentials lower than −65 mV and were silent.

**GABA and glutamate mediate rapid synaptic responses to SFO stimulation**

To assess the nature of synaptic transmission from SFO to MnPO, we applied electrical stimulation at the ventral edge of the SFO, a location likely to activate the majority of axons coursing toward MnPO. Postsynaptic responses were evident in 28/40 neurons tested. Since these featured both excitatory and inhibitory components, their further characterization was conducted in the presence of selective pharmacological receptor antagonists. In ACSF containing NBQX (5 \(\mu\)M) and APV (20 \(\mu\)M), SFO stimulation evoked IPSPs at a latency of 8.9 ± 1.2 ms, reversing polarity at −65 mV and reversibly blockable on addition of BIC (20 \(\mu\)M; \(n = 10\); Fig. 1, A and B). In any given neuron, IPSPs displayed a constant latency over a range of stimulation intensities and ability to follow three pulses at 20 Hz, features deemed consistent with a monosynaptic connection. In ACSF containing BIC, SFO stimulation evoked excitatory postsynaptic potentials (EPSPs) at a latency of 7.9 ± 1.3 ms \((n = 7)\). EPSPs also displayed constant latencies over a range of stimulation intensities and reliably followed three pulses at 20 Hz.

At resting membrane potentials, EPSPs displayed fast and slow components. The amplitude of the “slow” component measured at 200 ms after the stimulus artifact was reduced by membrane hyperpolarization and by the addition of APV (20 \(\mu\)M; Fig. 1C; \(n = 10\), consistent with an \(N\)-methyl-\(d\)-aspartate (NMDA) receptor-mediated component. The remaining fast component of the EPSP was abolished by further addition of NBQX (5 \(\mu\)M; Fig. 1C). Under voltage clamp in the presence of ACSF containing NBQX and APV, SFO-evoked inhibitory postsynaptic currents (IPSCs; \(V_{th} = −45\) mV) were recorded as outward currents that reversed at −66.5 ± 3.5 mV \((n = 11)\) and were blockable with BIC \((n = 3)\). IPSC amplitudes (but not rise-time or decay time constants) differed significantly \((P < 0.01)\) depending on the cell’s location: 38.3 ± 11.5 pA for cells recorded in the dorsal MnPO \((n = 8)\) versus 16.8 ± 1.8 pA for cells in the ventral MnPO \((n = 20)\). In ACSF containing BIC, SFO-evoked excitatory postsynaptic currents (EPSCs; \(V_{th} = −55\) mV) were similar among ventral \((38.9 ± 8.8\) pA; \(n = 15\)) and dorsal MnPO cells \((33.5 ± 12.6\) pA; \(n = 4)\) and blockable with a cocktail of NBQX and APV \((n = 4)\). Thus under our experimental conditions, amino acids appear to be the sole mediators of rapid neurotransmission along the SFO pathway to MnPO.
all seven cells responded with a slow membrane depolarization, triggering a burst of action potentials (data not shown). After addition of NBQX to block AMPA and kainate receptors, 5/7 cells still responded to the stimulus barrage with slow membrane depolarization lasting up to 2 s (Fig. 1D). Since this response was either totally abolished or substantially attenuated after further addition of APV, it would appear that NMDA receptors mediated most or all of this slow depolarization.

**GABA<sub>B</sub> receptor activation suppresses SFO inhibitory and excitatory inputs to MnPO**

The preceding observations imply that GABA<sub>A</sub> and glutamate ionotropic receptors mediate virtually all rapid neurotransmission from SFO to MnPO. Two recent reports also identified MnPO with moderate to high levels of GABA<sub>B</sub>R1 immunoreactivity and mRNA (Bischoff et al. 1999; Margeta-Mitrovic et al. 1999), so we next investigated a role for GABA<sub>B</sub> metabotropic autoreceptors. Within minutes of the addition of a GABA<sub>B</sub> receptor agonist baclofen (0.3–10 μM) to the bath, both inhibitory and excitatory SFO-evoked postsynaptic currents underwent a significant concentration-dependent and reversible reduction in their amplitudes (Fig. 2).

In 0.3 μM baclofen, the mean IPSC amplitude was reduced to 70.8 ± 9.6% of control (n = 4; P < 0.01); in 3 μM baclofen, the reduction was to 32.4 ± 5.1% of control (n = 11; P < 0.01). Similarly, for EPSCs, 0.3 μM baclofen reduced their mean amplitude to 81 ± 9.6% of control (n = 4; P < 0.05), 3 μM baclofen effected a reduction to 39.3 ± 5.6% of control (n = 6; P < 0.01), and 10 μM baclofen caused a reduction to 16.3 ± 7.5% of control (n = 3; P < 0.001). Given that baclofen can also influence postsynaptic membrane conduc-

![FIG. 1. Subfornical organ (SFO) stimulation evokes amino-acid mediated inhibitory and excitatory postsynaptic potentials in median preoptic nucleus (MnPO) neurons. A: traces of current-clamp recordings in control artificial cerebrospinal fluid (ACSF) containing 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo [f] quinoxaline-7-sulfoamide disodium (NBQX; 5 μM) and (R)-2-amino-5-phosphonovaleric acid (APV; 20 μM) indicate that a single stimulus at the ventral edge of SFO (arrow) evokes an inhibitory postsynaptic potential (IPSP) that is blocked by subsequent addition of bicuculline (BIC; 20 μM). B: summary plot from 6 neurons show the membrane potential dependence of IPSP amplitudes. Inset: series of voltage traces obtained during intracellular current pulses. Note the voltage dependence of the SFO-evoked IPSP delivered during current pulse and the low threshold potential on return to resting membrane potentials from the most hyperpolarized pulses. C: traces from another MnPO neuron recorded in ACSF containing BIC (Control) reveal that an excitatory postsynaptic potential (EPSP) follows the SFO stimulation artifact. Note reduction in the late EPSP component after addition of APV, indicative of a significant contribution from N-methyl-D-aspartate (NMDA) subtype receptors. Subsequent addition of NBQX blocks all evoked responses. D: in media containing BIC and NBQX, the top trace (Control) from another MnPO cell shows that a 20-Hz burst stimulation in SFO evokes a prolonged envelope of depolarization, sufficient to trigger a series of action potentials. Bottom trace shows blockade of this response on addition of APV, suggesting that NMDA receptors alone are sufficient to mediate this SFO-evoked response.](http://jn.physiology.org/)

Because the activity of SFO neurons is reported to vary from slow firing in vivo (0.1–1.2 Hz; Tanaka et al. 1987) to bursting patterns when recorded in vitro as cell preparations (Washburn et al. 2000), we were prompted to test responsive MnPO neurons to SFO stimulation presented in a brief burst (20 Hz for 0.5 s). When applied in the presence of bicuculline,
stances (see Fig. 5), we examined the kinetics of normalized IPSCs and EPSCs and found no changes (Fig. 2). While barium (1 mM) does influence postsynaptic GABA_B receptor function, its presence did not influence baclofen’s ability to suppress SFO-evoked IPSCs (n = 4 cells) or EPSCs (n = 3 cells). Efforts to evaluate the influence of conotoxin were impeded by its ability to block synaptic transmission. Baclofen-induced effects on IPSCs (n = 3) or EPSCs (n = 2) were completely blockable when CGP52432 (1–3 μM), a potent GABA_B receptor antagonist (Lanza et al. 1993), was added prior to application.

GABA_B inhibition of SFO-evoked neurotransmission involves presynaptic mechanisms

To determine whether the baclofen-induced reduction in SFO-evoked IPSCs and EPSCs was mediated by a presynaptic mechanism that reduced transmitter release, we examined effects on paired-pulse protocols. SFO-evoked postsynaptic currents displayed varying degrees of paired-pulse depression (PPD), and less commonly facilitation (PPF), when tested at interstimulus intervals between 100 and 200 ms. In 19 cells, where a mean P2/P1 ratio of 0.81 ± 0.02 for paired IPSCs reflected a strong PPD, this ratio changed from a trend toward an increase in 0.3 μM baclofen (from 0.88 ± 0.05 to 1.04 ± 0.25; n = 4; P > 0.05) to a significant increase in 3 μM baclofen (from 0.92 ± 0.04 to 1.16 ± 0.06; n = 8; P < 0.01). This was due to baclofen’s suppression of the amplitude of both the P1 and P2 responses, with the larger action affecting the P1 response (see Fig. 3A). For EPSCs, an overall average P2/P1 ratio of 1.08 ± 0.08 (n = 15) included four cells displaying PPF (1.36 ± 0.19) and six cells displaying PPD (0.75 ± 0.04). Among the cells displaying PPD, the P2/P1 ratio changed from 0.82 ± 0.14 to 1.01 ± 0.27 (n = 4; P > 0.05) in 0.3 μM baclofen, and 1.52 ± 0.32 (n = 5; P < 0.05) in 3 μM baclofen, again reflecting an action on both P1 and P2 responses with the greatest change involving the amplitude of the P1 response. These features are consistent with a modulating action of presynaptic GABA_B receptors.

To test whether ambient levels of GABA in the extracellular space might be sufficient to activate GABA_B receptors, we assessed whether any changes would arise through blocking these receptors by application of CGP52432 for 6–10 min. We detected no significant change in holding current or input conductance (from 1.69 ± 0.26 to 1.71 ± 0.25 nS; n = 8), amplitude of SFO-evoked IPSCs (from 15.51 ± 6.44 to 15.45 ± 7.8 pA; n = 4 cells) or EPSCs (from 25.22 ± 7.59 to 24.28 ± 7.08 pA; n = 4) in the paired IPSC P2/P1 ratio (from 0.85 ± 0.09 to 0.97 ± 0.11; n = 4) or paired EPSC P2/P1 ratio (from 0.74 ± 0.16 to 0.68 ± 0.09; n = 2; P > 0.05 in all instances).

GABA_B receptor depression of TTX-independent transmitter release: mechanisms

MnPO neurons display spontaneous inhibitory and/or excitatory amino acid-mediated postsynaptic potentials. Investigation of the effects of baclofen on calcium- and TTX-independent miniature events that represent quantal release of transmitter can provide insight into their signaling mechanisms. In ACSF containing APV and NBQX, mIPSCs were amplified using a CsCl-based internal solution. As noted by Lenz et al. (1997), such a recording arrangement significantly reduced the outward current induced by 10 μM baclofen compared with data obtained using a potassium-based internal solution (7.8 ± 2.6 pA, n = 5, vs. 17.4 ± 2.1 pA, n = 18; P < 0.05). Under these conditions, addition of baclofen decreased mIPSC frequency to 31.7 ± 7.04% of control (from 1.3 ± 0.25 to 0.39 ± 0.12 Hz; P < 0.05) without affecting their amplitude distribution (9.57 ± 4.12 vs. 8.47 ± 4.04 pA, P > 0.05; n = 6; Fig. 4, A and B), rise, or decay times. Baclofen also significantly reduced mEPSC frequency (from 1.8 to 0.3 Hz; P < 0.05) without a change in their amplitude (n = 3; data not shown). Interestingly, in 2/4 cells tested, application of CGP52432 alone induced an increase in mIPSC frequency (161.9 ± 1.2% of control) without affecting amplitude distribution, rise, or decay times. Therefore by contrast with the negative findings on evoked responses mentioned above, ambient levels of GABA may be sufficient to activate some presynaptic GABA_B receptors under resting conditions in this preparation.

Depression of transmitter release by presynaptic GABA_B receptors is widely considered to be mediated primarily through modulation of calcium and/or potassium channels (Misgeld et al. 1995). As noted below in the section on postsynaptic GABA_B receptors, barium and ω-conotoxin GVIA alter receptor coupling to potassium and calcium channels, respectively. However, when tested for an influence on baclofen-induced actions on mIPSCs, each agent alone nonsignificantly increased mIPSC frequency, but neither altered the response to baclofen (Fig. 4B). To evaluate possible coupling to high-voltage–activated (HVA) calcium channels other than the N-type, we applied cadmium at a concentration (100 μM) that completely blocked postsynaptic calcium channels in MnPO neurons (see Kolaj and Renaud 2001). The result was a nonsignificant increase in mIPSC frequency (to 115.8 ± 8% of control). Cadmium did significantly decrease mIPSC amplitude (to 69.6 ± 6.3% of control; n = 5; P < 0.001; Fig. 4B) and decay times (from 37.13 ± 1.01 to 33.06 ± 2.06 ms; P < 0.05), an action likely due to its effect on postsynaptic GABA_A receptors (LeFeuvre et al. 1997).
Postsynaptic GABA<sub>B</sub>-mediated responses in MnPO neurons

Baclofen depressed excitability in 80% of cells tested in the dorsal and ventral MnPO. Under current clamp, applications of baclofen (5–10 μM) induced a gradual membrane hyperpolarization (12.8 ± 1.1 mV) that peaked after 30–150 s and lasted 5.6 ± 1.1 min, accompanied by a cessation in spontaneous action potential discharges (Fig. 5A; n = 14 cells). When recorded under voltage clamp (V<sub>H</sub> = −55 mV) and in the presence of 0.5 μM TTX, the response to baclofen was a slowly rising outward current that persisted for 7.1 ± 0.8 min at the 10 μM concentration (Fig. 5B; n = 14 cells). This response was concentration-dependent (EC<sub>50</sub> = 0.36 μM), associated with a proportionate increase in membrane conductance (110.7 ± 3.9% with 0.3 μM baclofen; 139.3 ± 7.1% with 10 μM baclofen; P < 0.05), and was completely blockable in the presence of CGP52432 (1–3 μM; n = 13). Whereas the addition of GTP-γ-S to the internal solution yielded responses to 10 μM baclofen that were similar in magnitude to those under control conditions (peak outward current of 16.3 ± 3.6 pA and conductance increase of 142.1 ± 9.1%; n = 4), the effect was not reversible by 20 min. These data are consistent with mediation through G protein–coupled receptors.

In hippocampus and other preparations, electrical stimulation may evoke sufficient release of GABA to reveal a late slow IPSC due to activation of postsynaptic GABA<sub>B</sub> receptors, an event that can be enhanced by addition of GABA uptake inhibitors (Isaccson et al. 1993). When tested in the presence of NO711 (25–50 μM) or guvacine (50–100 μM) to block GABA uptake, and in ACSF containing GABA<sub>A</sub> and glutamate receptor blockers, no late slow IPSCs were observed to follow a

**FIG. 4.** Baclofen attenuation of miniature (m)IPSC frequency is resistant to potassium and calcium channel blockers. A: top: continuous control traces show mIPSCs recorded with a cesium-based internal solution (see METHODS) in the presence of NBQX, APV, and TTX. Note the reduction in mIPSC frequency in the presence of baclofen (10 μM for 50 s). Bottom: a cumulative interspike interval probability graph on the left confirms a significant reduction (Kolmogorov-Smirnov sample test, P < 0.001) but no significant change in mIPSC amplitude on the right (P > 0.05). B: summary histograms on the left show effects of barium (1 mM), α-conotoxin GVIA (1 μM), and cadmium (100 μM) on the baclofen-induced (10 μM) reduction in mIPSC frequency. On the right, summary histograms reflect corresponding mIPSC amplitudes. Number of cells for each category is shown in graph legend. Each blocker was added 3–6 min before co-administration with baclofen for 50 s.

**FIG. 5.** Baclofen acting at postsynaptic GABA<sub>B</sub> receptors induces membrane hyperpolarization and outward current in MnPO neurons. A: whole cell current-clamp recording from a MnPO neuron (resting membrane potential, −55 mV) shows a slowly developing, prolonged, and reversible membrane hyperpolarization associated with cessation of action potential firing in response to a 50-s application of baclofen (open bar). B: whole cell voltage-clamp recording from another neuron in the presence of TTX shows a baclofen-induced slowly rising outward current (V<sub>H</sub> = −55 mV). C: dose–response relationships show the concentration dependence of the baclofen-induced outward current (numbers of tested cells in brackets).
burst of SFO stimuli (5–10 pulses at 20 Hz; \( n = 5 \)). In contrast with the situation in hippocampal slice preparations and despite the evidence for functional GABA<sub>B</sub> receptors in MnPO, it would appear that GABA released by SFO stimulation was not sufficient to activate postsynaptic GABA<sub>B</sub> receptors. Other investigators have reported difficulties in evoking postsynaptic GABA<sub>B</sub> responses (e.g., Jensen et al. 2003; Overstreet and Westbrook 2001). In MnPO, one might speculate that this reflects a combination of the extrasynaptic location of GABA<sub>B</sub> receptors and the particular anatomical features of the synapses arising from SFO afferents. Other identified inputs to MnPO neurons have yet to be examined.

**Postsynaptic GABA<sub>B</sub> receptors increase a potassium conductance**

The baclofen-induced membrane hyperpolarization and increased conductance in MnPO neurons was mediated by potassium channels, consistent with data from other CNS neurons (Misgeld et al. 1995). \( I-V \) plots revealed intersections and/or net baclofen-induced conductances that reversed at \(-97.7 \pm 2.6 \text{ mV} \) (Fig. 6; \( A-C \); \( n = 16 \)), approximating the potassium equilibrium potential \( (E_K) \) of \(-98 \text{ mV} \) under these experimental conditions. In ACSF containing 10 mM potassium, this value shifted from \(-93.2 \pm 1.7 \) to \(-63.3 \pm 5.4 \text{ mV} \) (Fig. 6D; \( P < 0.01; n = 6 \)), a \( 29.8 \pm 6.1 \text{ mV} \) difference consistent with the calculated \( E_K \). Conductance plots also displayed inward rectification, with a coefficient for the net baclofen-induced current (calculated as the ratio between currents at \(-110 \) and \(-20 \text{ mV} \)) increasing significantly from \(-1.27 \pm 0.38 \) in control ACSF to \(-6.40 \pm 1.11 \) in ACSF containing 10 mM potassium \( (P < 0.05; n = 6) \); virtually all of this difference was attributable to increased currents at hyperpolarized levels. The addition of barium \((0.1–1 \text{ mM})\) to block potassium channels induced an inward current of \(-5.6 \pm 1.8 \text{ pA} \) coupled with a decrease in membrane conductance \((80.3 \pm 5.6\% \text{ of control})\), with reversal of polarity at \(-90.3 \pm 5.6 \text{ mV} \). In the presence of barium, reductions were noted in both the baclofen-induced outward current (Fig. 6E; from \(15.2 \pm 2.3 \) to \(3.6 \pm 1.3 \text{ pA} \); \( P < 0.001 \)) and conductance \((130.8 \pm 4.6\% \) to \(107 \pm 4.1\% \text{ of control}; P < 0.05; n = 5)\). The baclofen-induced outward current was unchanged by further addition of cadmium \((200 \mu \text{M}; n = 3)\).

Because of variability among cells in the amplitude of the baclofen-induced outward currents, we examined whether this might correlate with specific intrinsic membrane properties. In data obtained with \(3 \mu \text{M} \) baclofen \((n = 42)\), the current amplitude did correlate with resting membrane conductance \((\text{Pearson correlation coefficient } R = 0.404; P < 0.01)\), and with the amplitude of a hyperpolarizing-activated time-dependent rectification \((R = 0.548; P < 0.001)\). The latter, common among MnPO neurons (Bai and Renaud 1998a,b), was identified as the hyperpolarizing-activated cationic current \( I_h \) based of its blockade in 3/3 cells on addition of a specific inhibitor \(ZD7288\) \((50 \mu \text{M}; \text{Harris and Constanti 1995})\). However, this treatment failed to significantly alter the baclofen-induced outward current \((13 \pm 1.9 \text{ pA} \text{ in control vs. } 11.8 \pm 2.9 \text{ pA in } ZD7288)\) or resting conductance \((125 \pm 9.6\% \text{ in control vs. } 118.3 \pm 5.9\% \text{ in } ZD7288)\). Thus whereas the membrane hyperpolarization evoked by baclofen appears to be more pronounced in cells with a strong \( I_h \), the baclofen-induced current is largely due to opening of potassium channels of the GIRK type.

**Postsynaptic GABA<sub>B</sub> receptors suppress a calcium conductance**

Activation of GABA<sub>B</sub> receptors is also known to suppress calcium conductances (Misgeld et al. 1995). We therefore used cesium-filled pipettes, added TTX to the ACSF, and replaced calcium with barium as a charge carrier, a choice based on
barium’s ability to block both the baclofen-induced activation of GIRK channels as well as several other potassium channels. With neurons held at −60 mV and exposed to slow depolarizing ramp commands (from −90 to +50 mV), the evoked calcium current had a mean amplitude of −274 ± 43 pA (n = 9), peaking between −10 and 0 mV. Cadmium (100 μM), a broad-spectrum calcium channel blocker, virtually abolished this current (1.8 ± 0.6% of control; n = 3). As illustrated by the example in Fig. 7, application of 10 μM baclofen reversibly suppressed peak inward current to 70.4 ± 3.6% of control (n = 9; P < 0.01). Baclofen also produced a small outward current (4.5 ± 2.2 pA; n = 9), but its contribution to calcium currents was negligible after using leak subtraction protocol.

In an earlier study on dissociated MnPO neurons, we reported that activation of norepinephrine α2 receptors selectively suppressed N-type HVA calcium channels (Kolaj and Renaud 2001). When tested on four cells (see example in Fig. 7A), we observed a comparable suppression in the HVA calcium current with both 10 μM baclofen (65.9 ± 6.5%) and 30 μM norepinephrine (68.4 ± 9.3%), but saw no additive effect when baclofen and norepinephrine were administered together (71.8 ± 6.2%; P > 0.05). After application of ω-conotoxin GVIA (2 μM), a specific blocker of N-type calcium channels that irreversibly suppressed peak calcium currents to 67.9 ± 4.6% (P < 0.05), baclofen had no further effect, implying that GABA_B receptors and α2 adrenoceptors in MnPO neurons both act to suppress N-type HVA calcium channels.

**Postsynaptic GABA_B receptors modulate I_{AHP} and increase cell excitability**

Physiological consequences of Ca^{2+} entry through Ca^{2+}-dependent channels include the activation of Ca^{2+}-dependent K⁺ currents that underlie and contribute to action potential repolarization, afterhyperpolarization (AHP), and spike-frequency adaptation (Bevan and Wilson 1999; Meech 1978). Given the suppressant effect of baclofen on Ca^{2+}-channels, we examined membrane currents underlying the AHP (I_{AHP}) under voltage clamp. Tail currents following 200-ms depolarizing voltage steps (to +10 mV from a holding potential of −55 mV) were depressed by baclofen (10 μM) from 41.1 ± 12.6 to 28 ± 9.9 pA (i.e., to 64.9 ± 4.9% of control; P < 0.01; Fig. 7, B and C) in 8/10 cells tested. As expected, I_{AHP} was also significantly reduced by barium (1 mM; to 50.9 ± 14.9% of control) and by ω-conotoxin GVIA (2 μM; to 12.2 ± 5.2% of control). The nonselective Ca^{2+} channel blocker cadmium (200 μM) reduced I_{AHP} to 5.1 ± 1.9% of control (Fig. 7, B and C).

Since AHP can have a profound influence on the firing pattern of a neuron (Storm 1990), we next examined how baclofen might affect the firing pattern of MnPO neurons based on response to a test pattern consisting of 2-s depolarizing current pulses of 35 pA prior to, and during, the peak of the baclofen-induced response. To offset the hyperpolarizing effect of baclofen, current was injected to maintain the cell at the resting potential level (51.4 ± 1.9 mV in control vs. 50.9 ± 1.6 mV during baclofen; 5 μM; n = 13). As shown in the example in Fig. 8, A and B, application of such tests at the peak of the baclofen-induced response revealed both an increase in firing frequency (118.4 ± 5.5% over control; P < 0.05) and a shortening of the mean interval (1st 5) instantaneous frequencies (123.1 ± 4.4% over control; P < 0.01). In contrast, no change was detected in the last five instantaneous frequencies in the burst (103.9 ± 7%). Therefore spike frequency adaptation (calculated as the ratio between the initial and last interspike frequencies) increased in the presence of baclofen (from 1.71 ± 0.25 to 2.03 ± 0.32; P < 0.05). There was no obvious correlation between the size of baclofen-induced outward current and changes in these firing properties. Analysis of first spike during the 35-pA pulse in 13 cells revealed no significant change in amplitude (60.1 ± 4.1 mV in control vs. 60.3 ± 4 mV in baclofen; measured from threshold), width (2.35 ± 0.24

**FIG. 7.** Baclofen suppresses N-type high-voltage activated (HVA) calcium currents and currents underlying the afterhyperpolarization (AHP). A: data from a single experiment show time course of the peak HVA-calcium current evoked by ramp protocol (inset, from −90 to +50 mV). Note the reversible baclofen (Bac) and norepinephrine (NE) effects and their occlusion when applied together. The baclofen effect is completely blocked by preincubation with ω-conotoxin GVIA (ω-CTX). Cadmium eliminates all HVA calcium currents. B: whole cell patch recordings show outward tail currents (I_{AHP}) underlying the AHP. Each trace is the average of 5 consecutive responses to 200-ms depolarizing voltage steps to +10 mV from a holding potential of −55 mV applied every 20 s. In data obtained from the same MnPO cell, note the reduction in amplitude in the presence of baclofen (5 μM) or cadmium (100 μM). C: summary histogram shows the effects of barium (1 mM), ω-conotoxin GVIA (1 μM), and cadmium (100 μM) on the baclofen-induced (5 μM) reduction in I_{AHP}. Each blocker was added 3–6 min before co-administration with baclofen for 50 s.
or spike delay (26.4 ± 3.6 vs. 27.4 ± 4.8 ms; measured as time from the beginning of pulse to the 1st peak). However, in keeping with the data for $I_{AHP}$ presented above, there was a significant decrease in the amplitude of the AHP (14.7 ± 1.6 vs. 12.3 ± 1.5 mV; $P < 0.05$), supporting a role of the AHP in defining the firing properties of these neurons. In cadmium, which blocked calcium channels and decreased $I_{AHP}$, mean firing frequency increased by 179.5 ± 24.9% during a 35-pA pulse ($P < 0.05$), and the AHP decreased from 15.4 ± 3.5 to 11.6 ± 3.1 mV ($P < 0.05$; $n = 4$), without a change in spike amplitude, width, or threshold.

**DISCUSSION**

In this investigation, we examined the nature of rapid neurotransmission from the SFO to MnPO, a pathway deemed critical for behavioral (e.g., drinking) and/or autonomic (e.g., cardiovascular) responses to agents in the circulation (e.g., angiotensin). In slice preparations, data from patch-clamp re-
cordings in MnPO revealed that electrical stimulation in the SFO evoked rapid monosynaptic inhibitory and excitatory postsynaptic responses mediated by GABA<sub>A</sub> and glutamate AMPA and/or NMDA receptors, respectively. NMDA receptor subtypes alone could mediate the major component of responses elicited by a train of stimuli applied to SFO. Activation of GABA<sub>B</sub> receptors with baclofen attenuated inputs to MnPO neurons in a manner consistent with an action at presynaptic autoreceptors, suppressing both SFO-evoked IPSCs and EPSCs, attenuating SFO-evoked paired-pulse depression ratios, and suppressing the frequency of TTX-independent mIPSCs and mEPSCs without altering their kinetics. Baclofen’s effects on miniature events were insensitive to potassium and calcium channel blockers, contrasting with their ability to attenuate events mediated through postsynaptic GABA<sub>B</sub> receptors. Features of the latter, noted in 80% of MnPO neurons tested, included a baclofen-induced TTX-resistant, G protein–mediated activation of an inwardly rectifying potassium conductance and suppression of an N-type HVA calcium conductance. Figure 9 offers a simplified schematic of these observations. Baclofen’s pre- or postsynaptic effects were reversibly blocked by prior application of CGP52432. However, lack of change in membrane conductances, SFO-evoked currents, or paired-pulse ratios during a prolonged application of CGP52432 alone suggested that ambient levels of GABA under our experimental conditions were insufficient to activate most pre- or postsynaptic GABA<sub>B</sub> receptors. The exception was a CGP52432-induced increase in mIPSC frequencies observed in a small sample of MnPO neurons.

**Amino acids, the mediators of rapid transmission in SFO inputs to MnPO**

In earlier extracellular studies in vivo, Tanaka et al. (1987) reported that stimulation in SFO evokes inhibitory and excitatory responses in MnPO neurons. The present intracellular analysis convincingly demonstrates mediation of rapid neurotransmission in this pathway by GABA<sub>A</sub> and glutamate receptors. Interestingly, SFO-evoked excitation in MnPO neurons has been proposed to be mediated by the peptide angiotensin (Tanaka et al. 1987). Consistent with this notion are reports of angiotensin-like immunoreactivity in SFO neurons and their axonal projections (Jhamandas et al. 1989; Lind et al. 1984), a high-density of angiotensin AT<sub>1</sub> receptors, in MnPO (Lenkei et al. 1998), and activation of a population of MnPO neurons by exogenous angiotensin (Bai and Renaud 1998a; Travis and Johnson 1993). It seems reasonable to propose angiotensin as a coexisting neuropeptide whose co-release might contribute to “delayed amplification” of synaptic transmission (see Hökfelt et al. 2000). If so, one might anticipate that SFO stimulation might evoke both an early event related to the release of rapidly acting neurotransmitters and a “later prolonged” increase in neuronal excitability due to the effects of a co-released neuropeptide. In fact, such has been observed in vivo in extracellular recordings from hypothalamic supraoptic nucleus neurons, also known targets of SFO efferent fibers, where the late component of the SFO-evoked response could be partially reduced by local application of saralasin (Jhamandas et al. 1989). Conceivably, the late response was the “signature” of endogenous angiotensin release in an SFO efferent pathway. In vitro slice preparations may present conditions (e.g., room temperature; need for peptidase inhibitors) less favorable for eliciting/detecting endogenous release of the peptide. Notably few in vitro mammalian brain slice preparations have in fact yielded observations attributable to their synaptic release. It is anticipated that future studies will clarify the features related to endogenously released angiotensin within the SFO projection to MnPO.

**Presynaptic GABA<sub>B</sub> receptors: a calcium independent mechanism?**

Activation of presynaptic GABA<sub>B</sub> receptors can reduce GABA and/or glutamate release, thereby reducing the efficacy of neurotransmission at many CNS sites; this is commonly attributed to inhibition of presynaptic calcium channels (reviewed in Misgeld et al. 1995), although other mechanisms have been implicated (Capogna et al. 1996). Our observations concur with presynaptic GABA<sub>B</sub> receptor modulation at both inhibitory and excitatory inputs to MnPO neurons, including those arising from SFO. In the present situation, it is interesting that the baclofen-induced effects lacked sensitivity to barium,

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**FIG. 9.** Simplified schema to summarize the present observations. **Top:** glutamatergic (GLU) and GABAergic (GABA) pathways arising from SFO as one component of rapid neurotransmission to MnPO neurons, mediated by ionotropic glutamate (GLU-R) and GABA<sub>A</sub> (GABA<sub>A</sub>-R) receptors. **Bottom:** MnPO neurons receive additional glutamatergic and GABAergic afferents whose origins remain to be identified (?). Rapid glutamatergic and GABAergic transmission to MnPO from SFO and other afferents may be negatively modulated by presynaptic metabotropic GABA<sub>B</sub> receptors (GABA<sub>B</sub>-R). Also depicted are GABA<sub>B</sub>-Rs located on MnPO neurons, whose activation may affect neuronal excitability through increase in inwardly rectifying potassium conductances (GIRK) and/or reduction in N-type or potassium-activated calcium conductances.
GABA \(_B\) receptors couple with potassium and calcium channels

In contrast with observations in hippocampus where \( \text{GABA}\(_B\)\) receptors can mediate a late slow IPSP (reviewed in Misgeld et al. 1995), the SFO-evoked IPSPs in MnPO neurons were completely blockable with bicuculline. In hippocampus, postsynaptic \( \text{GABA}\(_B\)\) receptors are located extrasynaptically, and their activation is under tight control of GABA uptake mechanisms (Scanziani 2000). While the latter was not investigated in detail here, effects of their activation by exogenously applied agonists resembled that reported in other neurons: suppression of neuronal firing consequent to membrane hyperpolarization through increase in a G protein–coupled inwardly rectifying potassium conductance and suppression of voltage-gated calcium channels. The latter action of \( \text{GABA}\(_B\)\) receptor activation has been noted to differ among neurons in terms of channel subtypes involved: L-, N-, and P/Q-type in hippocampal inhibitory neurons (Lambert and Wilson 1996); N- and P/Q-type in supraoptic neurons (Harayama et al. 1998); and L-type in cerebellar granule cells (Wojcik et al. 1990). Observations in MnPO neurons are consistent with a selective effect on N-type channels since the magnitude of the baclofen-induced reduction of calcium channel function was comparable with that induced by \( \omega\)-conotoxin GVIA, and most of the baclofen-induced suppression was completely abolished by \( \omega\)-conotoxin GVIA. In acutely dissociated MnPO neurons, we previously reported suppression of N-type channels by norepinephrine acting via \( \alpha_2\) adrenoceptors (Kolaj and Renaud 2001). Given that the action of baclofen in these slice preparations could be occluded by norepinephrine (Fig. 7), it is possible that similar downstream mechanism(s) may mediate suppression of N-type calcium channels subsequent to activation of both \( \text{GABA}\(_B\)\) and \( \alpha_2\) adrenoceptors.

In MnPO neurons, a correlation observed between the magnitude of the baclofen-induced outward current and the resting conductance suggest a possible contribution of this current to resting membrane conductance. On the other hand, we saw no correlation between baclofen-induced suppression of SFO-evoked responses and the change in resting conductance. Collectively, these findings suggest a lack of correlation between the presynaptic and postsynaptic effects of baclofen, in keeping with the evidence that pre- and postsynaptic \( \text{GABA}\(_B\)\) receptors in MnPO neurons utilize different mechanisms and/or receptor subtypes, as proposed for other central sites (Yamada et al. 1999). Interestingly, it has been suggested that presynaptic \( \text{GABA}\(_B\)\) receptors contain R1a subunits while the postsynaptic receptors contain R1b subunits (Bischoff et al. 1999; Kaufmann et al. 1998a,b).

**\( \text{GABA}\(_B\)\) receptors: a role in modulation of firing patterns?**

The \( \text{GABA}\(_B\)\) receptor-induced decrease in neuronal excitability and spontaneous firing was seen to involve but a few tens of picamps of outward current. However, because of the high-input resistance (low resting conductance) of MnPO neurons (~1 nS), this could result in a significant change in membrane potential (10 pA will cause a change of 10 mV with a conductance of 1 nS), especially important if the membrane potential were close to threshold for action potential generation. The fact that cadmium did not change the amplitude of the baclofen-induced outward (potassium) current implies little contribution of calcium channels toward the magnitude of membrane hyperpolarization. However, should the calcium currents measured in our configuration be of somatic origin, it is conceivable that they may couple with calcium-activated potassium channels that contribute toward the magnitude of the AHP whose reduction could lead to increased firing rate and longer burst duration during transient depolarization (Matsushima et al. 1993). Furthermore, we did not find correlation between baclofen-induced outward current and change in firing properties suggesting heterogeneous coupling between \( \text{GABA}\(_B\)\) receptors and potassium versus calcium channels. By analogy with recent observations in retinal ganglion cells (Zhang et al. 1998), it is possible that \( \text{GABA}\(_B\)\) receptors could serve as discriminators, effectively reducing the influence of weak signals while boosting responses to strong signals. In this regard, one might speculate that SFO neurons might be able to provide such strong signals to MnPO neurons, as might arise under conditions where threats to body fluid homeostasis (e.g., raised plasma osmolality or circulating levels of angiotensin) would raise impulse traffic in the SFO to MnPO pathway.

**GRANTS**

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