Direct Actions of Carbenoxolone on Synaptic Transmission and Neuronal Membrane Properties

Kenneth R. Tovar, Brady J. Maher, and Gary L. Westbrook
Vollum Institute, Oregon Health and Science University, Portland, Oregon

Submitted 21 January 2009; accepted in final form 10 June 2009

Tovar KR, Maher BJ, Westbrook GL. Direct actions of carbenoxolone on synaptic transmission and neuronal membrane properties. J Neurophysiol 102: 974–978, 2009. First published June 17, 2009; doi:10.1152/jn.00060.2009. The increased appreciation of electrical coupling between neurons has led to many studies examining the role of gap junctions in synaptic and network activity. Although the gap junction blocker carbenoxolone (CBX) is effective in reducing electrical coupling, it may have other actions as well. To study the non–gap junctional effects of CBX on synaptic transmission, we recorded from mouse hippocampal neurons cultured on glial micro-islands. This recording configuration allowed us to stimulate and record excitatory postsynaptic currents (EPSCs) or inhibitory postsynaptic currents (IPSCs) in the same neuron or pairs of neurons. CBX irreversibly reduced evoked α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor–mediated EPSCs. Consistent with a presynaptic site of action, CBX had no effect on glutamate-evoked whole cell currents and increased the paired-pulse ratio of AMPA and N-methyl-D-aspartate (NMDA) receptor–mediated EPSCs. CBX also reversibly reduced GABA_A receptor–mediated IPSCs, increased the action potential width, and reduced the action potential firing rate. Our results indicate CBX broadly affects several neuronal membrane conductances independent of its effects on gap junctions. Thus effects of carbenoxolone on network activity cannot be interpreted as resulting from specific block of gap junctions.

INTRODUCTION

Electrical coupling can synchronize the activity of coupled neurons. Although the properties of gap junction proteins such as connexins and their expression in neurons are now well documented (Connors and Long 2004; Nagy et al. 2004), the impact of electrical coupling on neuronal ensembles or networks is still being explored. Although synchronization of membrane voltage by action potentials (Landisman et al. 2002) or subthreshold depolarizations (Long et al. 2004) can distribute and propagate concerted signals in electrically coupled networks, network activity involves a complex mixture of receptors, channels and intrinsic membrane properties. Thus elucidating the role of gap junctions and electrical coupling requires tools that reliably and specifically block gap junctions. One of the most commonly used gap junction blockers is the glycyrretinic acid derivative carbenoxolone (CBX). CBX eliminates electrical and gap junctional coupling in several experimental systems (Martin et al. 1991; Schoppa and Westbrook 2002; Zsiros and Maccarelli 2005). However, in our prior experiments in brain slices, CBX seemed to alter synaptic transmission independently of gap junction block (Schoppa and Westbrook 2002). Here, we examined whether CBX directly affected synaptic transmission and action potential firing by using whole cell voltage- and current-clamp recording in mouse hippocampal neurons cultured on glial micro-islands.

METHODS

Cell culture

Micro-island cultures were prepared using the method of Bekkers and Stevens (1991). Round glass coverslips (15 mm; Bellco Glass) were coated with 0.15% agarose, allowed to dry, and sprayed with a solution containing poly-lysine (0.1 mg/ml) and collagen (0.375 mg/ml) in 17 mM acetic acid. Hippocampi dissected from P0 to P1 mice (C57BL/6) were incubated in a papain solution (200 units; Worthington Biochemical) for 30 min (35°C), and the papain was inactivated in complete media containing bovine serum albumin (2.5 mg/ml) and trypsin inhibitor (2.5 mg/ml). The tissue was triturated with fire-polished Pasteur pipettes, and individual cells in suspension were counted using a hemocytometer. To make glial micro-islands, cells were plated at 100,000 cells per 35-mm dish (Nunc). To grow neurons on prepared glial micro-islands, cells were plated at 25,000 cells per 35-mm dish. All cultured cells were grown in 3 ml of complete media. Media contained 5% heat-inactivated fetal bovine serum (Lonza). Media was replaced weekly by removing 1.5 ml of media per dish and replacing it with the same volume of freshly prepared media.

Electrophysiology

For whole cell voltage-clamp recordings, we used neurons that were 6–15 days in vitro (DIV). Micro-islands were perfused by gravity flow with extracellular solution containing (in mM) 168 NaCl, 2.4 KCl, 10 HEPES, 10 glucose, and 1.3 CaCl_2 (pH 7.4; 320 mOsm). Recording electrodes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) and had resistances of 2–5 MΩ. Recording electrodes were filled with a solution containing (in mM) 140 K-gluconate, 6.23 CaCl_2, 8 NaCl, 2 MgCl_2, 10 EGTA, 10 HEPES, 2 Na_ATP, and 0.1 Na_2GTP (pH 7.4; 320 mOsm). All recordings were at room temperature using Axopatch 1C amplifiers and Axograph acquisition software (AxographX, Sydney, Australia). Series resistance was always <10 MΩ and was compensated by ≥80% by the amplifier circuitry. Data were low-pass filtered at 5 kHz and acquired at 10 kHz. The membrane voltage was held at −70 mV. Brief (0.5–1 ms) depolarizations to 20 mV triggered unclamped action potentials in the axon, and evoked voltage-clamped synaptic currents in the dendrite. Neurons were stimulated at low frequency (0.1–0.133 Hz).

For current-clamp experiments, we first patched onto the neuron in voltage-clamp mode and determined whether the neuron was excitatory or inhibitory by evoking postsynaptic currents as described above. We then bathed the recorded neuron in NBQX (5 μM), t-AP5 (100 μM) or D-CPP (5–10 μM), and SR 95531 (10 μM) to block AMPA, NMDA, and GABA_A receptors, respectively. For current-headed experiments, we used the voltage-clamp configuration.

Address for reprint requests and other correspondence: K. R. Tovar, Vollum Inst., L474, Oregon Health and Science University, 3181 SW Sam Jackson Park Rd., Portland, OR 97239 (E-mail: tovarkr@ohsu.edu).
Carbenoxolone was marginally less effective than 100 μM when comparing groups of cells, increasing the CBX concentration from 50 to 100 μM CBX on an individual cell did not produce further inhibition (Fig. 1C, right). To test whether CBX had a direct postsynaptic effect on AMPA receptors, we applied glutamate (500 μM) to block AMPA receptors by brief (0.5 ms) depolarizations of the soma. Increasing concentrations of CBX applied sequentially to a neuron reduced the EPSC. The arrowhead indicates the action current (rendered in gray). CBX effect occurs quickly. Once CBX was applied, reduction occurred within the 7.5-s interstimulus interval. CBX did not reduce whole cell glutamate–activated currents (95.7 ± 1.2% of the control amplitude, n = 5) indicating that CBX did not directly inhibit AMPA receptors. The above results suggest that the reduction of the AMPA receptor–mediated EPSC involves a presynaptic mechanism. Consistent with this possibility, CBX increased the paired-pulse ratio (PPR) of AMPA receptor–mediated EPSCs. The ratio of the second EPSC to the first (P2/P1; 50-ms interstimulus interval) was 1.38 ± 0.12 in CBX (100 μM) compared with 1.19 ± 0.08 in control (P < 0.01, n = 17; Fig. 2, A and B). 50 μM CBX also increased the AMPA PPR (0.94 ± 0.07 in control; 1.05 ± 0.06 in CBX, n = 14, P < 0.005; data not shown). As expected for a presynaptic mechanism, the extent of block of the EPSC by CBX (100 μM) correlated with its effect on the PPR (r = 0.78). However, there was no relationship between the control PPR and the extent of block (r = 0.23 for 100 μM CBX and r = 0.02 for 50 μM CBX), indicating that all the synapses were susceptible to CBX inhibition.

AMPA and NMDA receptors co-localize at most postsynaptic sites in central neurons (Bekkers and Stevens 1989; Jones and

---

**FIG. 1.** Carbenoxolone (CBX) reduces α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor–mediated excitatory postsynaptic currents (EPSCs). A: In this autaptic culture, we evoked AMPA receptor mediated EPSCs by brief (0.5 ms) depolarizations of the soma. Increasing concentrations of CBX applied sequentially to a neuron reduced the EPSC. The arrowhead indicates the action current (rendered in gray). B: CBX at 100 μM produced a greater decrease in AMPA receptor–mediated EPSCs than 50 or 50 μM (ANOVA, P < 0.05). C: CBX effect occurs quickly. Once CBX was applied, reduction occurred within the 7.5-s interstimulus interval. D: CBX did not reduce whole cell currents evoked by direct application of glutamate. In A and D, black traces are control recordings; red traces indicate recordings from the same cell in the presence of the indicated concentration of CBX. Recordings were done in d-AP5 (100 μM) or d-CPP (10 μM) to block N-methyl-d-aspartate (NMDA) receptors.
Effects on action potentials and repetitive firing

To evoke synaptic currents, we used brief depolarizing voltage injections that triggered an unclamped action potential in the axon. In the soma, the resulting action current was followed by an evoked synaptic current (Fig. 1A). In the presence of CBX, we noticed a consistent increase in the width of the action currents. We examined this issue in greater detail by evoking action potentials in current clamp in the presence of AMPA, NMDA, and GABA<sub>A</sub> receptor antagonists.

Consistent with our observations using voltage clamp, CBX increased the width of action potentials evoked by current pulses by 11.4 ± 1.9% (n = 7; P < 0.005; 50 μM) and 24.5 ± 2.9% (n = 8; P < 0.00005; 100 μM; Fig. 4, A and B). During long current injections, action potentials in control and CBX gradually accommodated (Fig. 4C). However, neurons in CBX fired fewer action potential during a 200-ms current injection. The reduction in the number of action potentials was 1.8 ± 0.48 (n = 7; P < 0.01) for 50 μM CBX and 2.6 ± 0.57 (n = 9; P < 0.01) for 100 μM CBX (Fig. 4D). In our experiments, 10 μM CBX did not affect action potential width or the number of action potentials (data not shown). As shown in Fig. 4E, the interval between the first and second spike was increased to 56.1 ± 13.8 ms in 100 μM CBX compared with 27.4 ± 4.2 ms in control (n = 7; P < 0.05). The effects on spike width and the repolarization potential are consistent with a reduction of potassium conductances that repolarize the membrane. CBX (100 μM) also decreased the resting membrane input resistance by 20.6% (134.9 ± 15.1 MΩ control compared with 107.1 ± 12.5 MΩ in CBX, n = 24; P < 0.001). Thus in excitatory hippocampal neurons, CBX affects membrane conductances that are active at the resting membrane potential as well as voltage-dependent potassium conductances involved in action potential repolarization.

**DISCUSSION**

Our results showed that the commonly used gap junction blocker CBX has a broad range of non–gap junctional actions, including a reduction in excitatory and inhibitory synaptic currents, attenuation of membrane repolarization and spike rate, and decreases in input resistance. We focused on excitatory neurons from the mouse hippocampus, a relatively homogenous cell group. However, CBX had similar actions on GABAergic interneurons, strongly suggesting that our results are not limited to specific cell types.

We applied CBX in the same concentration range necessary to block gap junctions. CBX affected synaptic transmission at concentrations as low as 10 μM, whereas the effects on action potentials required ≥50 μM. Cultured neurons generally provide greater access to drug applications than brain slices. For example, the non–gap junctional actions of CBX occurred within a few seconds in our experiments. Equilibration is slower in brain slices, but the concentration at neuronal membranes should reach similar levels within the tens of minutes used in brain slice perfusion of drugs. CBX is membrane permeant (Monder et al. 1989) and will diffuse readily across membranes should reach similar levels within the tens of minutes used in brain slice perfusion of drugs. CBX is membrane permeant (Monder et al. 1989) and will diffuse readily across membranes.
membranes, perhaps aiding access but delaying recovery. Thus this drug cannot be reliably used to indicate a role for gap junctions in complex physiological responses such as network activity. Although CBX generally reduced excitatory transmission, the reduction in inhibitory transmission could lead under appropriate circumstances to net increases in network activity.

The molecular basis of the broad effects of CBX is not clear. CBX (18-β-glycyrrhetic acid sodium hemisuccinate) is a water-soluble derivative of a compound from licorice root, which has a steroid-like structure (Davidson and Baumgarten 1988). Other steroid-like molecules can duplicate the effect of CBX on gap junctions (Davidson and Baumgarten 1988; Davidson et al. 1986), although neurosteroids like aldosterone (50 μM), prednisolone (100 μM), hydrocortisone (100 μM), or cholesterol (100 μM) did not block gap junctions in cultured human fibroblasts (Davidson et al. 1986). The incomplete reversibility of AMPA receptor–mediated EPSC inhibition likely reflects a large number of nonspecific membrane binding sites that slow CBX clearance. Calcium channels are the most likely presynaptic target causing the reduction in release probability in excitatory neurons. For example, CBX can block calcium channels in retinal cone photoreceptors (Vessey et al. 2004). Our results confirm and extend prior reports of gap junction–independent effects of CBX on membrane properties of hippocampal neurons (Rekling et al. 2000; Rouach et al. 2003). Although Rouach et al. (2003) saw no effect of CBX on the frequency of miniature EPSCs, our results clearly indicate that CBX can affect evoked release of glutamate. Rouach et al. (2003) reported a reversible effect of CBX on spike threshold, whereas only the block of GABA_A IPSCs was completely reversible in our experiments. Our results also indicate that the actions of CBX cannot be attributed to one type of ion channel.

CBX is effective in blocking gap junctions when assessed in isolation. However, our results and those of others (Chepkova et al. 2008; Rekling et al. 2000; Rouach et al. 2003; Vessey et al. 2004) indicate that CBX acts on multiple membrane conductances and thus should not be used to assess the role of gap junctions on network activity. Given the gap junction–independent effects of CBX, studies that used CBX exclusively to infer the role of gap junctions in epileptiform activity (Gigout et al. 2006; Jahromi et al. 2002; Ross et al. 2000; Traub et al. 2001), pain (Spataro et al. 2004), cardiac pathology (Kojodjojo et al. 2006), and even synaptic transmission (Yang and Ling 2007) may need reassessment. Unfortunately other gap junction blockers also have effects on other channels (Cruikshank et al. 2004). The parent compound of CBX, glycyrrhizic acid, does not block gap junctions and thus has been used to test the specificity of CBX on network activity (Elsen et al. 2008; Rouach et al. 2003). However, glycyrrhizic acid also has non-gap junctional actions, which greatly complicates the assessment of network activity. Thus genetic ablation or dominant negative approaches (Hormuzdi et al. 2001; Placantonakis et al. 2006) should remain the standard for network studies in the absence of more specific gap junction blockers.

Acknowledgments

Present address of B. J. Maher: University of Connecticut, Physiology and Neurobiology, 75 North Eagleville Rd., Rm. 175, U-3156, Storrs, CT 06268.

Grants

This work was supported by National Institute of Neurological Disorders and Stroke Grant NS-26494 to G. L. Westbrook.

References


J Neurophysiol • VOL 102 • AUGUST 2009 • www.jn.org
FIG. 4. CBX alters action potential firing. Current injections (200–500 ms; 50–300 pA) in whole cell current clamp resulted in trains of action potentials. The action potentials in A are averaged aligned action potentials in control and CBX (100 µM) from a single neuron. B: pairwise comparison shows that CBX (50 and 100 µM) increased the action potential width at half-height (arrowhead in A). We used only the 1st action potential in the train for analysis. C: CBX (100 µM) reduced the number of action potentials and increased the interspike interval in the 1st 200 ms of depolarizing current injection (red traces) compared with control (black traces). The mean spike times for this neuron are shown below the voltage traces by the thick black vertical hash marks, whereas the individual spike times (25 repetitions per condition) are shown in thin vertical hash marks. D: a pairwise comparison shows the reduction in the number of spikes in the 1st 200 ms in CBX (50 and 100 µM) compared with control. E: CBX (100 µM) also increased the interspike interval of the 1st pair of action potentials in response to sustained current injections. Open red circles are 50 µM CBX; closed red circles are 100 µM CBX.


Jones KA, Baughman RW. Both NMDA and non-NMDA subtypes of glutamate receptors are concentrated at synapses on cerebral cortical neurons in culture. **Neuron** 7: 593–603, 1991.


