Post-Episode Depression of GABAergic Transmission in Spinal Neurons of the Chick Embryo

NIKOLAI CHUB AND MICHAEL J. O’DONOVAN
Section on Developmental Neurobiology, Laboratory of Neural Control, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892

Received 29 August 2000; accepted in final form 11 January 2001

Chub, Nikolai and Michael J. O’Donovan. Post-episode depression of GABAergic transmission in spinal neurons of the chick embryo. J Neurophysiol 85: 2166–2176, 2001. Whole cell recordings were obtained from ventral horn neurons in spontaneously active spinal cords isolated from the chick embryo [embryonic days 10 to 11 (E10–E11)] to examine the post-episode depression of GABAergic transmission. Spontaneous activity occurred as recurrent, rhythmic episodes approximately 60 s in duration with 10- to 15-min quiescent inter-episode intervals. Current-clamp recording revealed that episodes were followed by a transient hyperpolarization (7 ± 1.2 mV, mean ± SE), which dissipated as a slow (0.5–1 mV/min) depolarization until the next episode. Preliminary reports of this work have been published in abstracts (Chub and O’Donovan 1995, 1998b, 1999).

INTRODUCTION

Spontaneous activity is a property of many, perhaps all, networks in the developing nervous system. It comprises recurring episodes, containing one or many cycles of discharge, that are synchronized throughout the neurons of the active network (for review see O’Donovan 1999). This activity has been implicated in several aspects of network development (for review see Katz and Shatz 1996), but its genesis is not entirely understood. Recent modeling studies have argued that spontaneous activity can be produced by excitatory networks with activity-dependent synaptic depression (Senn et al. 1996; Tabak et al. 1999, 2000). In such models, the network is recruited through the positive-feedback excitation of recurrent synaptic connections. Once active, the network engages activity-dependent depression, which reduces functional network connectivity below a level that can sustain activity. The network then progressively recovers, allowing another episode of activity to occur (for reviews see O’Donovan and Chub 1997; O’Donovan et al. 1998).

In the chick embryo spinal cord, it is known that an episode of activity is followed by a prolonged depression of synaptic transmission in many spinal pathways (Fedirchuk et al. 1999), but the cellular mechanisms of this depression are still unknown. Ventrally located GABAergic neurons are an important source of synaptic drive to motoneurons during spontaneous episodes and have been implicated in the patterning of flexor and extensor motoneuron activity (Sernagor et al. 1995). In addition, it has been shown that GABAergic networks are capable of supporting spontaneous activity in the embryonic day 10 to 12 (E10–12) cord following excitatory amino acid blockade (Chub and O’Donovan 1998a) or in the E4–5 cord following cholinergic blockade (Milner and Landmesser 1999). Given the importance of GABAergic networks in early spinal cord development, in this paper we have characterized GABAergic transmission and its modulation by spontaneous activity. Preliminary reports of this work have been published in abstracts (Chub and O’Donovan 1995, 1998b, 1999).

METHODS

Experiments were performed on the isolated spinal cord of E10–11 White Leghorn chicken embryos. The dissection procedures have

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
been described in detail previously (Landmesser and O’Donovan 1984; O’Donovan 1989). In short, the lumbosacral cord was isolated and cooled to 10–15°C in Tyrode’s solution containing (in mM) 139 NaCl, 5 KCl, 17 NaHCO<sub>3</sub>, 3 CaCl<sub>2</sub>, and 12 glucose, equilibrated with 95% O<sub>2</sub>-5% CO<sub>2</sub> to pH 7.3–7.4. An isolated section of the lumbosacral cord (LS1–LS7) was then transferred to a recording chamber and superfused at room temperature (20–22°C). Recordings were made after heating the bath solution to 28°C. During both the dissection and the recording, the isolated cord was continuously superfused with Tyrode’s solution equilibrated with O<sub>2</sub>-CO<sub>2</sub>. The dissection and the recording, the isolated cord was continuously superfused with Tyrode’s solution equilibrated with O<sub>2</sub>-CO<sub>2</sub>

Recordings were made from ventral horn neurons located in spinal segments LS1–LS3 that contain a significant portion of GABAergic neurons at E10–12 (Antal et al. 1994). The whole cell recording (“blind patch” technique) (see Blanton et al. 1989) was used as modified for the isolated spinal cord of the chick embryo (Chub and O’Donovan 1998a; Sernagor and O’Donovan 1991). Micropipettes were pulled from thin-walled glass capillaries (TW 100-3, World Precision Instruments) in two stages using a Brown-Flaming puller (P-80/PC, Sutter Instrument). Tip resistances with intracellular solution were between 4 and 6 MΩ (measured in the extracellular solution).

Initial current-clamp experiments were performed using an intracellular solution that contained (in mM) 10 NaCl, 130 K-glucosate, 10 HEPES, 1.1 EGTA, 1 MgCl<sub>2</sub>, 0.1 CaCl<sub>2</sub>, 1 Na<sub>2</sub>ATP, and standard extracellular (Tyrode’s) solution. For this, we added the Gramicidin-perforated patch, we used an intracellular solution containing (in mM) 140 KCl, 10 NaCl, 10 HEPES, and 30 sucrose. Gramicidin D (Dubos, Sigma) was dissolved in methanol (2 mg in 100 µl) and added to the pipette solution at final concentration of 50 µg/ml. The tip of the micropipette was back-filled with gramicidin-free patch solution. Sealing, perforating (up to 30–40 min), and identification of the neurons were all made in standard extracellular (Tyrode’s) solution. For measurements of E<sub>GABA<sub>a</sub></sub> (perforated patch configuration, voltage-ramp protocol) we added blockers of various voltage-activated and GABA<sub>a</sub> conductances to the extracellular solution which then contained (in mM) 139 NaCl, 5 KCl, 17 NaHCO<sub>3</sub>, 1 MgCl<sub>2</sub>, 1 MnCl<sub>2</sub>, 12 glucose, 10 tetraethylammonium acetate (TEA), 5 CsCl, 0.001 tetrodotoxin citrate (TTX), and 0.1 2-hydroxysaclofen (2-HS), pH 7.3–7.4.

Miniature synaptic postsynaptic currents (mPSCs) were recorded from cells under whole cell voltage clamp (at V<sub>hold</sub> = −70 mV) that exhibited a good seal resistance (>5–7 GΩ) and a high-input resistance (0.6–1.2 GΩ). For these measurements, we used an intracellular solution containing (in mM) 10 NaCl, 94 K-glucosate, 36 KCl, 10 HEPES, 1.1 EGTA, 1 MgCl<sub>2</sub>, 0.1 CaCl<sub>2</sub>, 1 Na<sub>2</sub>ATP, and 0.5 Na-GTP. For long-duration measurements of the agonist-evoked I<sub>GABA<sub>a</sub></sub> (in whole cell configuration), the ATP concentration was increased to 5 mM. In addition, to reduce the possible confounding effects of the intracellular Ca<sup>2+</sup> elevation that accompanies spontaneous episodes (O’Donovan et al. 1994) and that may lead to changes of I<sub>GABA<sub>a</sub></sub> because of Ca-dependent receptor phosphorylation (Mozzyns and Cherubini 1998), we used an intracellular solution with a high concentration of the Ca<sup>2+</sup> buffer [11 mM bis-(o-aminophenoxy)-N,N,N’,N’-tetraacetic acid (BAPTA) instead of 1.1 mM EGTA]. Thus for these experiments, the whole cell solution contained (in mM) 84 K-glucosate, 46 KCl, 10 HEPES, 11 BAPTA, 1 MgCl<sub>2</sub>, 0.1 CaCl<sub>2</sub>, 5 Na<sub>2</sub>ATP, and 0.5 Na-GTP. In the final set of the experiments, we determined the effects of spontaneous episodes on E<sub>GABA<sub>a</sub></sub>. In these experiments, the voltage-dependent channel blockers could not be added to the bath solution because this prevented the generation of episodes. Therefore we added to the intracellular solution (containing BAPTA) the following channel blockers: 10 mM QX-314 (lidocaine, N-ethyl bromide quaternary salt), 10 mM TEA, and 0.1 mM verapamil ([±]-verapamil, methoxy-HCl). All intracellular solutions were adjusted to pH 7.2 with KOH.

Membrane potential was recorded with an Axoclamp 2A amplifier, current with an Axopatch 200B amplifier (Axon Instruments) and ventral root activity by using tight-fitting plastic suction electrodes coupled to DAM70 DC amplifiers (World Precision Instruments). Signals were filtered and amplified (DC – 2 or 5 kHz, Cyber Amp 380), digitized (DigiData 1200), and stored on the hard disk of an IBM-compatible computer using AxoScope 1, Clampex 6, or 7 software (Axon Instruments). E<sub>GABA<sub>a</sub></sub> was measured by applying a quasi-stationary voltage-ramp command generated by Clampex 6 and analyzed off-line with Clampfit 6 software. The amplitude and inter-event intervals of mPSCs were measured off-line with Mini Analysis Program 3.01 (Jaejin Software) and analyzed with Origin 4.1 (Microcal Software), Sigma Plot 3.0, and SigmaStat 2.0 (Jandel). Access resistance (series resistance) was not compensated. Typically, it was not more than 40 MΩ (in perforet patch recording) and 25 MΩ in whole cell configuration that would give an error in voltage command of up to 2.7 mV at V<sub>hold</sub> = −30 mV (perforated patch) and up to 2.5 mV at V<sub>hold</sub> = 0 mV for whole cell recording.

Local application of drugs was performed as described earlier (Sernagor et al. 1995). In brief, drugs were dissolved in Tyrode’s solution and then pressure-applied (10–15 psi) from a micropipette similar to that used for whole cell recording. The injection micropipette was first positioned inside the ventral horn, and then the recording electrode was introduced. A whole cell recording was made from a cell located as close as possible to the injection side. The drugs for local and bath application were prepared as 10-mM stocks in distilled water and diluted to the final concentration in the extracellular solution immediately before use or made up fresh and used immediately. Drugs were obtained from Sigma (St. Louis, MO) with exception of TTX 2-HS, 6-cyano-7-nitroquinolinic acid-2,3-dione disodium (CNQX), and bicuculline-methiodide (BIC), which were obtained from RBI (Research Biochemicals International, Natick, MA).

Liquid junction potentials were measured as described by Neher (1992) with 3 M KCl agar bridges, well equilibrated (2–3 days) in Tyrode’s solution. They were not more than 3–4.5 mV for our intracellular solutions. Data were not corrected these errors. Results are expressed as means ± SE.

**RESULTS**

Inter-episode changes of membrane potential and the hyperpolarizing effect of bicuculline during the inter-episode interval

Whole cell recordings were made from ventral horn neurons in spontaneously active isolated spinal cords of the chick embryo (E10–11). Spontaneous activity appeared as repetitive, rhythmic episodes nearly 60 s in duration with 10 to 15-min inter-episode intervals. Long-lasting recordings (up to 1 h in current-clamp configuration) revealed that spinal neurons were transiently hyperpolarized after an episode, and this recovered as a slow depolarization until the next episode. Figure 1 shows examples of the membrane potential trajectory of two ventral spinal neurons during, and in the interval between, spontaneous episodes. Approximately 1–2 min after an episode, the membrane became hyperpolarized by −6 to −10 mV (−6.5 ± 1.2 mV, mean ± SE, 8 neurons) and then slowly depolarized (0.5–1 mV/min) until the next episode. In some cells, the inter-episode depolarization reached threshold and triggered several spikes (Fig. 1, dashed arrow). Otherwise, neurons did not fire between episodes.

Earlier preliminary observations (Chub and O’Donovan 1995) had suggested that the inter-episode depolarization was affected by the GABA<sub>a</sub> antagonist bicuculline. This was consistent with indirect evidence in the chick spinal cord that GABA was depolarizing (Sernagor et al. 1995). Therefore we tried to test the dependence of the inter-episode membrane potential on...
GABAergic conductances, we pressure-applied the GABAa antagonist bicuculline (100 \mu M, n = 7) at various times after spontaneously occurring episodes (20 s, 4 and 8 min) and measured the accompanying changes in membrane potential ($V_m$) and input resistance ($R_{in}$). The drug was most effective when it was applied 8 min after an episode, close to the time when the drug was applied 20 s after the episode. In this experiment, bicuculline was applied to the neuron at 3 min before a spontaneous episode, approximately midway through the inter-episode interval, resulting in a smaller membrane hyperpolarization than that at 8 min (Fig. 2B, middle and left).

These findings suggested that the slow inter-episode depolarization were caused, in part, by endogenous GABA. Consistent with a role for GABA in the inter-episode depolarization, we found that low concentrations of bicuculline (5 \mu M; 3 experiments) strongly and reversibly lengthened the inter-episode interval from 11.8 ± 1 min to 51.3 ± 4.7 min. Following wash out of the drug, the intervals partially recovered to 25.1 ± 5.2 min (2 h wash out).

**Depolocidin GABAa equilibrium potentials measured with gramicidin–perforated-patch recording**

To confirm the depolarizing nature of GABA on ventral spinal neurons, we used perforated-patch recording with gramicidin D as the voltage-insensitive cation ionophore (Hladky and Haydon 1984; Kyrozis and Reichling 1995; Reichling et al. 1994). In the gramicidin–perforated-patch configuration, the resting membrane potential ($V_m$) of spinal neurons varied between $-50$ and $-70$ mV ($-60 ± 3$ mV, $n = 10$). Neurons were distinguished from glial cells by their ability to fire action potentials.

When a neuron was identified, voltage-dependent channel blockers and 2-HS were included in the Tyrode’s solution to block voltage-activated and GABAb conductances, respectively (see methods). GABAb conductances were blocked because we wanted to establish the current-voltage relationship ($I-V$) and the equilibrium potential for GABAa receptor activation ($E_{GABAa}$). This extracellular solution also blocked spontaneous episodes. The $I-V$ of the GABA-evoked currents ($I_{GABAa}$) was determined by applying a slow (80 mV/s) triangular voltage ramp to the neuron (Fig. 3A, top). Local application of 100 \mu M GABA at holding potential ($V_{hold}$) $-50$ mV evoked a long-lasting inward current (Fig. 3A, bottom) ranging from $-58$ to $-276$ pA ($-106 ± 37$ pA, 6 neurons). For the neuron shown in Fig. 3A, $V_m$ was $-50$ mV (estimated from the zero current crossing under control conditions), and $E_{GABAa}$ was $-30$ mV (estimated from the intersection of the $I-V$ curves generated under control conditions and during GABA application; Fig. 3B). $I_{GABAa}$ was linear in the range of holding potentials from $-60$ to $0$ mV (Fig. 3C, as determined by subtraction of the control current from the current recorded in the presence of GABA). The mean $E_{GABAa}$, determined from six perforated-patch recordings, was $-29.1 ± 2.9$ mV and varied from $-37$ to $-18$ mV (Fig. 3E). It should be noted that inclusion of the channel blockers in the external solution decreased the resting potential of the neuron by $5-10$ mV, but $E_{GABAa}$ was always about $20$ mV more positive than the resting $V_m$.
In five neurons, the recording was converted into the whole cell configuration by applying gentle suction. Under these conditions, $I_{GABA}$ was measured no earlier than 3 min after membrane rupture to allow adequate time for somal dialysis. In the example shown in Fig. 3D, $I_{GABA}$ was measured in gramicidin–perforated-patch configuration as in C, and then the neurons patched membrane was ruptured to obtain the whole cell configuration. The voltage ramps and GABA application were repeated to obtain measurements of the $I-V$ relation in the whole cell configuration with symmetrical intracellular and extracellular Cl– solutions. Note the shift of $E_{GABA}$ from $-21$ mV in the gramicidin–perforated-patch recording to 0 mV in the whole cell configuration, consistent with Cl– as the charge carrier for $I_{GABA}$.

Quantal and persistent GABAa currents during the inter-episode interval

mPSCs were recorded in whole cell voltage-clamp configuration using an electrode chloride concentration (48.2 mM, $E_{Cl} = -30$ mV) close to the intracellular [Cl–] estimated from the perforated-patch recordings. The mPSCs were observed in ventral horn neurons but not in glial cells and were analyzed only during the inter-episode interval (not during spontaneous episodes). The mPSCs ranged from $-4$ to $-190$ pA and had variable kinetics with an average rise time of 3.5 ms (measured between 10 and 90% of peak amplitude) and a slower, approximately monoexponential decay, $t = 26.1$ ms ($V_{hold} = -70$ mV, 3 neurons, 154 events). These parameters were in general similar to the parameters of mPSCs recorded in cultured chick spinal neurons (O’Brien and Fischbach 1986) and from other types of embryonic spinal neuron (Ali et al. 2000; Gao et al. 1998; Rohrbough and Spitzer 1999).

To establish whether generation of the mPSCs required action potentials, we determined the effects of TTX on the amplitude and frequency of the events. We found that the mPSCs persisted following bath application of 1 mM TTX (3 experiments; Fig. 4), which also abolished spontaneous episodes and action potentials (data not shown). To establish the effects of TTX on the mPSCs, we measured their frequency and amplitude during a 2-min period before and 5–8 min after application of the TTX. All measurements were done at a $V_{hold} = -70$ mV. The mean amplitude of the mPSCs was 13.3 pA in control conditions (476 events), and this decreased to...
11.5 pA after 5–8 min in TTX (458 events). The corresponding averaged inter-event intervals were 743.5 ms in control and 771.5 ms in TTX. Both the amplitude and frequency of mPSCs in TTX were not statistically significantly different from the controls (ANOVA on Ranks, Dunn’s Method; compared in 3 neurons). Representative examples of averaged mPSCs are illustrated in Fig. 4B and are shown superimposed (obtained during 2 1-min time period in control and in TTX). These findings suggest that the great majority of mPSCs occurring during the inter-episode interval arise from action-potential-independent transmitter release. However, just before an episode, some neurons do start to fire so that some of the mPSCs occurring at this time will arise from action potentials.

To establish the transmitters responsible for generation of the mPSCs, we bath-applied receptor antagonists in the presence of 1 μM TTX. The blockers were added in the following sequence: for the first 15 min we added the glutamate antagonists 2-amino-5-phosphonopentanoic acid (AP5; 50 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX; 10 μM). Then we added the GABAa antagonist bicuculline (BIC; 25 μM) for an additional 15 min, followed for another 15 min by the addition of the glycine antagonist strychnine (10 μM). The AP5 and CNQX always reduced, but never completely abolished, the mPSCs (Fig. 5A). When bicuculline was added to the glutamate antagonists, the remaining mPSCs were completely abolished in four of seven cells. In the other three cells, strychnine abolished all of the remaining spontaneous events (Fig. 5A). The mean frequency of mPSCs measured in the presence of AP-5 and CNQX decreased to 45 ± 5.9% from 100% of the control. When bicuculline was added to the excitatory blockers, the mPSCs frequency decreased to 3% from the control level (Fig. 5B; 2-min periods were used for measurement in 7 neurons). These data suggest that glutamate and GABA are the major transmitters contributing to the generation of mPSCs and allow us to estimate that approxi-
mately 42% of the total mPSCs are mediated by action-potential–independent quantal release of GABA. The amplitude of the GABAergic mPSCs was measured in four neurons, which did not have glycinergic mPSCs. In these cells (recorded in the presence of AP5 and CNQX), subsequent application of bicuculline completely abolished the mPSCs demonstrating that they originated from GABAergic transmission. The amplitude of the GABAergic miniature currents ranged from 6 to 31 pA with a median value of 8 pA (571 events from 4 neurons; each cell was separately analyzed and then averaged).

We also found that bath application of bicuculline (in the presence of TTX and glutamatergic receptor antagonists) reduced the membrane noise (see Fig. 5A) and blocked an inwardly directed, tonic $I_{\text{GABA}_a}$ (Fig. 5C). The amplitude of the tonic $I_{\text{GABA}_a}$ was measured as shown in Fig. 5C and ranged from 4 to 18 pA with a mean of 9.6 ± 2.8 pA (n = 5). It is unlikely that the tonic $I_{\text{GABA}_a}$ arises because of summation of high-frequency quantal GABAergic release, because individual GABAergic mPSCs were clearly separated one from another and often completely decayed before the next event (Fig. 5A).

**Activity-dependent modulation of mPSCs**

The mPSCs recorded at $V_{\text{hold}} = -70$ mV were superimposed on a slow inward current (Fig. 6, dashed line on the top) that was presumably responsible for the slow inter-episode depolarization we recorded under current clamp (Fig. 1). The slow inward current was voltage dependent: at $V_{\text{hold}} = -70$ mV its peak value was 15–35 pA, but at $V_{\text{hold}} = -30$ mV (close to $E_{\text{GABA}_a}$) it was greatly reduced (data not shown). These observations are consistent with the idea that slow inter-episode depolarization is mediated by persistent activation of the GABAergic receptors.

We also found that mPSCs changed systematically during the inter-episode interval. Before a spontaneous episode, the amplitude of the mPSCs was highest, and they often comprised multi-peak events (Fig. 6A, bottom left, asterisks). After a spontaneous episode, the mPSCs amplitude declined (Fig. 6A, bottom right) and then progressively recovered throughout the inter-episode interval. We quantified these changes by comparing the mPSCs ($V_{\text{hold}} = -70$ mV) during 30-s intervals (indicated by the rectangles in the top panel of Fig. 6A) before and after spontaneous episodes. The cumulative distributions show that mPSC amplitude distribution is shifted to the left after an episode (Fig. 6B). We also found that the mPSC intervals increased after an episode, and this is illustrated by the right hand shift of their cumulative distribution (Fig. 6C).

Following an episode, the mPSCs mean amplitude decreased from 15.9 pA (1,904 events) to 11.3 pA (1,627 events), and the mPSCs interval increased from 156 ms (1,894 events) to 184 ms (1,617 events). The post-episode mPSC measurements were significantly different from the control, pre-episode mPSC measurements ($P < 0.001$, Mann-Whitney rank sum test; 10 measurements, 5 neurons). The percentage change of the mean mPSCs amplitude (~29%) was greater than that for the mPSCs interval (+18%).

**Post-episode depression of GABAergic-mediated current**

The time-dependent effects of locally applied bicuculline on the inter-episode depolarization, coupled with the post-episode depression of the mPSCs amplitude, raised the possibility that the postsynaptic response to GABA might be depressed after an episode and then recover during the inter-episode interval. To test this hypothesis, we measured the GABAergic currents evoked by local application of the GABA agonist isoguvacine (100 µM) or GABA (100 µM) onto a recorded neuron, before and after spontaneous episodes. To avoid initiation of an episode by the local application of the agonist, the duration and pressure of the puff were routinely adjusted for each cell and thereafter kept constant during the measurements on that cell. For these, and all following measurements, we used an intracellular solution (see METHODS) with a high concentration of ATP to minimize the time-dependent rundown of the agonist-evoked $I_{\text{GABA}_a}$ (Kapur et al. 1999).

Fig. 6. Spontaneous episodes depress both mPSCs and the persistent current. A: 20-min record, similar to those shown in Fig. 1, but recorded under voltage clamp at a holding potential of ~70 mV. The dashed line on the top of the record provides a baseline reference for the persistent inward current, which progressively increased during the inter-episode interval. Synaptic currents accompanying the spontaneous episode (arrows) are off-scale. The rectangles at the beginning of the top trace illustrate 30-s segments before and after an episode, which are expanded in the bottom panels to show the synaptic events clearly. Asterisks (bottom left) show events with multiple peaks, which usually occurred before an episode. B and C: cumulative distributions show the changes of mPSCs amplitudes and inter-event intervals before and after spontaneous episodes. The data for the cumulative distributions were obtained from 5 neurons (30-s measurement periods) before and after spontaneous episodes.
We found that the amplitude of the isoguvacine-evoked current measured at $V_{\text{hold}} = -60 \text{ mV}$ was transiently depressed 30 s after an episode and partly recovered by 2 min 30 s (Fig. 7A). To quantify this post-episode depression, we compared the amplitude of the currents evoked 40–50 s before and 30–40 s after an episode. The mean amplitude of the GABA-induced current decreased from a pre-episode value of $63.9 \pm 10.2 \text{ pA}$ to $49.6 \pm 9.4 \text{ pA}$ after the episode ($n = 17$, 11 neurons). Similarly, the isoguvacine-evoked current decreased from $194.5 \pm 27 \text{ pA}$ to $153.3 \pm 20.5 \text{ pA}$ after the episode ($n = 5$, 2 neurons). Both of these decreases were statistically significant ($P < 0.001$, paired t-test). We found no change in the normalized time course of the current decay (not shown) suggesting that the post-episode decrease of the evoked current was probably not a result of GABAa receptor desensitization.

We were concerned that the post-episode depression of the evoked $I_{\text{GABAa}}$ might be due, in part, to clamping the membrane potential during the episode. For this reason, in three control experiments, the voltage clamp was switched to current clamp during the episode (Fig. 7B), and the evoked $I_{\text{GABAa}}$ was measured as before under voltage clamp. We found that the currents were still depressed after an episode indicating that the post-episode decrease was not due to clamping the synaptic drive or holding the membrane potential constant during episodes of rhythmic activity.

![Fig. 7](image-url)

**Fig. 7.** The amplitude of GABA agonist–evoked currents is transiently reduced after an episode. A: isoguvacine-evoked currents recorded under voltage clamp ($V_{\text{hold}} = -60 \text{ mV}$) in a ventral horn neuron, before and after the occurrence of a spontaneous episode. Isoguvacine (100 $\mu$M) was puff-applied for 5 s as indicated by the bars above the record. The **bottom panel** shows the isoguvacine-induced currents adjusted to the same baseline level and at expanded time scale. Note that amplitude of the GABAa currents has partly recovered 2 min 30 s after the episode. B: control record to show that the depression of the isoguvacine currents does not depend on voltage clamping the cell during the episode (see text for details). In this experiment the GABAa currents (application of 100 $\mu$M isoguvacine, before and after episode) were measured under voltage clamp ($V_{\text{hold}} = -60 \text{ mV}$), but during the episode the recording was switched to current clamp (without current injection). Note the 2 calibrations: the membrane potential calibration immediately follows the recording, and the current calibration is displayed at the end of the record. The isoguvacine-evoked currents before and after the episode are shown superimposed to the right of the record.

**Changes of $E_{\text{GABAa}}$ after episodes**

Previous work in the chick embryo spinal cord has shown that ventral horn neurons receive a prolonged (up to 60 s) and strong GABAAergic synaptic drive during an episode of activity (Chub and O’Donovan 1998a; Sernagor et al. 1995), raising the possibility that intracellular Cl$^-$ redistribution might be responsible for the post-episode $I_{\text{GABAa}}$ changes. If so, then experimental manipulation of the direction of Cl$^-$ flux during the episode should be accompanied by corresponding changes in post-episode amplitude (increase or decrease) of the evoked $I_{\text{GABAa}}$. To change the direction of neuronal Cl$^-$ flux, the cell was clamped at one of three different $V_{\text{hold}}$ during the episode: 0 mV, in-flux of Cl$^-$; −60 mV, out-flux of Cl$^-$; −30 mV, $E_{\text{Cl}}$; minimal Cl$^-$ flux. The isoguvacine-induced pre- and post-episode currents (Fig. 8) were measured at $V_{\text{hold}} = -60 \text{ mV}$ as described above. To reduce the possible confounding effect of intracellular Ca$^{2+}$ elevation (during the episode), we used an electrode solution with a high concentration of the Ca$^{2+}$ buffer (11 mM BAPTA instead of 1.1 mM EGTA, see METHODS).

For episodes at $V_{\text{hold}} = -60 \text{ mV}$ (i.e., below $E_{\text{Cl}}$), the post-episode, isoguvacine-induced currents decreased as described previously (Fig. 7). By contrast, when the neuron was clamped during the episode at 0 mV (i.e., above $E_{\text{Cl}}$), the amplitude of the isoguvacine-induced currents increased by 18% (3 cells) after the episode. When the neurons were clamped at −30 mV (close to the estimated $E_{\text{Cl}}$), we observed a small 5% decrease (mean of the same 3 cells) in the amplitude of the evoked currents after the episode. These findings indicate that the direction of the intracellular Cl$^-$ flux during the episodes determines the amplitude and direction of the post-episode changes of the evoked GABAa current.

In the next set of experiments, we analyzed the changes of
changes of intracellular [Cl\textsuperscript{−}] because of dialysis. As a result, we may have underestimated the actual changes.

We also noticed that the I-V relationships of \( I_{GABAa} \) before and after the episode were not completely parallel when the cell was at \( V_{\text{hold}} = -60 \) mV during the episode (Fig. 9B). After the episode, the GABAergic conductance was lower than before the episode falling from 17.3 ± 2.9 to 13.4 ± 2.2 nS (\( n = 5 \), paired t-test \( P < 0.05 \); Fig. 10). By contrast, when the cell was at \( V_{\text{hold}} = 0 \) mV, the isoguvacine-evoked conductance was similar before and after the episode (13.4 ± 3.6 before, 12.9 ± 3.2 nS after; not statistically different at \( P < 0.05 \), Fig. 10). This finding raises the possibility that some voltage-dependent process in the postsynaptic cell is affecting the GABAa conductance (see Discussion).

**DISCUSSION**

In this study, we have demonstrated that evoked \( I_{GABAa} \) is depressed after an episode and recovers during the inter-episode interval. One of the important contributors to this depression is a change of intracellular [Cl\textsuperscript{−}] after an episode of spontaneous activity. We estimate that the intracellular [Cl\textsuperscript{−}] decreases from a pre-episode concentration nearly 50 mM to about 35 mM. This change is presumably due to the prolonged activation of action-potential–dependent GABAergic transmission during the episode (Chub and O’Donovan 1998a; Sernagor et al. 1995). In addition, our results establish that GABAa conductances are active during the inter-episode interval in E10–11 spinal neurons. These conductances appear to be activated by action-potential–independent GABAergic mPSCs and a tonic \( I_{GABAa} \).

*Depolarizing GABAergic transmission in the embryonic chick spinal cord*

In the present study, we employed gramicidin–perforated-patch recording, which avoids dialysis-induced changes in the intracellular [Cl\textsuperscript{−}] and divalent cation concentrations (Hladky
Using this approach, we found that \( E_{\text{GABA}} \) was distributed over a wide range (~37 to ~18 mV) with a mean of ~30 mV. A similar distribution of \( E_{\text{GABA}} \) was found recently in embryonic Xenopus spinal neurons using perforated-patch recordings (Rohrbough and Spitzer 1996) and in cell-attached recordings of GABAergic channels on spinal neurons isolated from E15 rat spinal cords (Serafini et al. 1995). Depolarizing GABA responses have also been observed in other developing networks including the hippocampus, cortex, and retina (Ben-Ari et al. 1989; Fischer et al. 1998; Yuste and Katz 1991).

Although the \( E_{\text{GABA}} \) is more positive than \( V_m \) at rest, activation of GABAergic conductances can be functionally inhibitory as well as functionally excitatory. In the E11 chick cord, flexor sartorius and extensor femorotibialis motoneurons both receive synchronous GABAergic, cholinergic, and glutamatergic inputs during spontaneous episodes, but generate an alternating pattern of discharge. The alternation arises because sartorius motoneuron discharge is interrupted in each cycle at the time of peak extensor discharge. The interruption in firing is mediated primarily by a shunting GABAergic conductance (O’Donovan 1989). Recently, it was proposed that the differences in the functional action of GABA may depend, in part, on whether the synaptic conductances are somatically or dendritically located (Sernagor et al. 1995).

**Tonic and quantal GABA release during the inter-episode interval**

Approximately 42% of the mPSCs we recorded during the inter-episode interval were mediated by quantal GABA release. The majority of these events were action potential independent because the mPSCs frequency and amplitude were not significantly changed after 5–8 min in the presence of TTX. Consistent with this conclusion, our current-clamp recordings showed that spinal neurons only fired during, and just before, spontaneous episodes.

We estimate that about 10 channels (range 8–40) are located postsynaptically at each GABAergic synapse. This number is derived from the mean amplitude of the GABAergic mPSCs recorded in presence of TTX, AP5, and CNQX (8 pA, see results) and recent data indicating that one quantum of GABA release can activate all the GABA receptors located at a single synapse (Nusser et al. 1997). In addition, the calculation assumes a single GABA channel conductance 26 pS (Yang and Zorumski 1989) and \( P_{\text{open}} \sim 0.8 \) for GABAergic mPSCs (Jones and Westbrook 1995; Newland et al. 1991). The estimated density of synaptic GABA receptors is approximately one-half that reported for mature neurons that have 10–60 GABA channels located at a single synapse (De Koninck and Mody 1994; Edwards et al. 1990; Salin and Prince 1996).

In addition to quantal release, we also found evidence for a tonic \( I_{\text{GABA}} \). We consider it unlikely that the tonic current arises from the summation of asynchronous mPSCs because individual GABAergic events (isolated under glutamate blockade) decayed without substantial fusion with each other. It seems likely that the tonic current is responsible, in part, for the membrane potential changes occurring during the inter-episode interval. In addition, several lines of evidence suggest that the slow inter-episode depolarization was the result of endogenous GABA acting on GABA receptors. First, local-application bicuculline during the inter-episode interval hyperpolarized spinal neurons, consistent with depolarizing action of GABA. Second, the tonic inward current we recorded during the inter-episode interval under voltage clamp was largely abolished when the cell membrane potential was clamped near \( E_{\text{GABA}} \) (~30 mV). We also found that bath application of bicuculline abolished a tonic inward current in the presence of AP5, CNQX, and TTX. A tonic \( E_{\text{GABA}} \) of the magnitude we measured would depolarize ventral horn neurons approximately 8 mV, consistent with the maximum amplitude of the bicuculline-induced hyperpolarization recorded under current clamp during the inter-episode interval.

Tonic inward currents were only abolished after 5–8 min in the presence of TTX. Consistent with this conclusion, our current-clamp recordings significantly changed after 5–8 min in the presence of TTX. It seems likely that the tonic current is responsible, in part, for the membrane potential changes occurring during the inter-episode interval. Although only 42% of spontaneous mPSCs were GABAergic, it seems reasonable to assume that the post-episode changes of \( E_{\text{GABA}} \) will also contribute to their modulation during the inter-episode interval. Whether or not this is the only factor contributing to...
their modulation is unknown. For instance, when the cell was voltage clamped at −60 mV, we found that the GABA\textsubscript{A} conductance was greater before an episode than after it, and this could contribute to the increased mPSCs amplitude before the episode. We also detected a small decrease in the frequency of spontaneous mPSCs after an episode. While a reduction in frequency might indicate decreased presynaptic release, it may be that the frequency change is a detection issue, secondary to decreased mPSCs amplitude.

The post-episode reduction in the Cl\textsuperscript{−} driving force is also likely to account, in part, for the transient post-episode hyperpolarization we recorded in spinal neurons. When bicuculline was applied shortly after an episode (20 s), it did not change the membrane potential, consistent with the post-episode reduction of \( E_{\text{GABA}_\text{A}} \). By contrast, application of bicuculline 4–8 min after the episode hyperpolarized the cell resting potential, which would be expected as \( E_{\text{GABA}_\text{A}} \) recovered and became more positive. However, the changes of \( E_{\text{GABA}_\text{A}} \) are unlikely to be the only mechanism responsible for the inter-episode membrane potential changes. Bicuculline applied shortly after an episode produced no effect on the membrane potential even though isoguvacine or GABA puff-applied at this time was capable of generating a significant current. One explanation for this apparent discrepancy would be that the extracellular concentration of GABA was lowest after the episode and gradually increased during the inter-episode interval.

Concluding remarks

In previous work, it was shown that GABAergic ventral root–evoked potentials are transiently depressed after an episode and recover during the inter-episode interval (Fedirchuk et al. 1999). The depression in this and other pathways has been implicated in the genesis of spontaneous episodes by developing networks in the chick cord (Tabak et al. 1999, 2000). The results of the present work suggest that chloride redistribution may be one factor contributing to the post-episode depression of ventral root–evoked synaptic potentials. However, two factors complicate determining the magnitude of the episode-induced changes of intracellular [Cl\textsuperscript{−}] under physiological conditions. First, the outward chloride flux during an episode may be higher in cells that are voltage clamped at −60 mV (as in most of the present experiments) than when the membrane potential during the episode is allowed to vary. To address this concern, we examined the post-episode depression of the evoked \( I_{\text{GABA}_\text{A}} \) when the voltage clamp was switched to current clamp during the episode (Fig. 7B). Under this condition, we still observed a post-episode depression in the amplitude of the isoguvacine-evoked current. The second concern arises because it was not possible to employ the gramicidin-patch recording to assess \( E_{\text{GABA}_\text{A}} \) before and after an episode. This was because GABA\textsubscript{B} and voltage-dependent channel blockers (necessary to isolate \( I_{\text{GABA}_\text{A}} \)) had to be added to the electrode because they prevented episode generation when added extracellularly. This precluded the use of the gramicidin patch. As a result, the actual chloride changes induced by the episode may have been underestimated because they would have been opposed by electrode dialysis. One way to address these problems in future experiments may be the use of non-invasive chloride imaging (Verkman 1990) to assess the episode-induced changes of intracellular chloride. Furthermore, if chloride redistribution is a significant factor in the post-episode depression of synaptic transmission in GABAergic pathways, then blocking inwardly directed chloride pumps should affect this modulation. Although the regulation of intracellular [Cl\textsuperscript{−}] in developing neurons is not well understood, it is likely that inwardly directed Cl\textsuperscript{−} transporters are important in maintaining an elevated intracellular [Cl\textsuperscript{−}] (Rivera et al. 1999; Sun and Murali 1999). In Xenopus spinal neurons the elevated Cl\textsuperscript{−} concentration of Rohon-Beard cells depends on a bumentamide-sensitive Na\textsuperscript{+}-dependent Cl\textsuperscript{−} co-transporter (Rohrbough and Spitzer 1996).

Experimental manipulation of intracellular chloride may also provide insight into the recovery of spontaneous activity that occurs in E10–12 cords following glutamatergic blockade (Chub and O’Donovan 1998a) and in E4–5 cords following cholinergic blockade (Milner and Landmesser 1999). At both ages, the reappearance of spontaneous activity depends on functional GABAergic networks. In E10–12 embryos, the re-summation of spontaneous activity was accompanied by an increase in the amplitude of spontaneous, presumably GABAergic synaptic currents and potentials (Chub and O’Donovan 1998a). If these increases are mediated by a progressive increase in intracellular chloride concentration due to the action of inwardly directed chloride pumps, then we would predict that the recovery should be abolished if the pumps are blocked.

The authors are grateful to Drs. Chris McBain and Peter Wenner for helpful and critical reading during the preparation of the manuscript.

REFERENCES


BricKley SG, Cull-Candy SG, and Farrant M. Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of GABA\textsubscript{A} receptors. J Physiol (Lond) 497: 753–759, 1996.

Chen QX and Wong RKS. Suppression of GABA\textsubscript{A} receptor responses by NMDA application in hippocampal neurons acutely isolated from the adult guinea-pig. J Physiol (Lond) 482: 353–362, 1995.


