Pentobarbital Enhances GABAergic Neurotransmission to Cardiac Parasympathetic Neurons, Which Is Prevented by Expression of GABA\(_A\) \(\epsilon\) Subunit

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**Background:** Pentobarbital decreases the gain of the baroreceptor reflex on the order of 50%, and this blunting is caused nearly entirely by decreasing cardioinhibitory parasympathetic activity. The most likely site of action of pentobarbital is the \(\gamma\)-aminobutyric acid type A (GABA\(_A\)) receptor. The authors tested whether pentobarbital augments the inhibitory GABAergic neurotransmission to cardiac parasympathetic neurons, and whether expression of the GABA\(_A\) \(\epsilon\) subunit prevents this facilitation.

**Methods:** The authors used a novel in vitro approach to study the effect of pentobarbital on identified cardiac parasympathetic preganglionic neurons in rat brainstem slices. The cardiac parasympathetic neurons in the nucleus ambiguus were retrogradely prelabeled with a fluorescent tracer and were visually identified for patch clamp recording. The effects of pentobarbital on spontaneous GABAergic synaptic events were tested. An adenovirus was used to express the \(\epsilon\) subunit of the GABA\(_A\) receptor in cardiac parasympathetic neurons to examine whether this transfection alters pentobarbital-mediated changes in GABAergic neurotransmission.

**Results:** Pentobarbital increased the duration but not the frequency or amplitude of spontaneous GABAergic currents in cardiac parasympathetic neurons. Transfection of cardiac parasympathetic neurons with the \(\epsilon\) subunit of the GABA\(_A\) receptor prevented the pentobarbital-evoked facilitation of GABAergic currents.

**Conclusions:** Pentobarbital, at clinically relevant concentrations, prolongs the duration of spontaneous inhibitory postsynaptic currents that impinge on cardiac parasympathetic neurons. This action would augment the inhibition of cardiac parasympathetic neurons, reduce parasympathetic cardioinhibitory activity, and increase heart rate. Expression of the GABA\(_A\) receptor \(\epsilon\) subunit in cardiac parasympathetic neurons renders the GABA receptors insensitive to pentobarbital.

**Material and Methods**

All animal procedures were performed in compliance with the institutional guidelines at George Washington University.
University (Washington, DC) and are in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association and the NIH publication Guide for the Care and Use of Laboratory Animals. Two anatomical markers were used in this study. The traditional fluorescent tracer rhodamine (XRITC) was used to identify cardiac parasympathetic neurons located in the nucleus ambiguus. Two adenoviruses were used either to just express green fluorescent protein (GFP) or to coexpress both GFP and the GABA $\alpha$ subunit in cardiac parasympathetic neurons.

**Labeling of Cardiac Parasympathetic Neurons and Surgical Procedures**

Preganglionic cardiac parasympathetic neurons synapse upon postganglionic cardiac parasympathetic neurons located at the base of the heart. In an initial surgery, Sprague-Dawley rat pups (4–10 days old) were anesthetized with methoxyflurane and exposed to hypothermia during the surgery (10–15 min) to slow the heart and aid in recovery. A right thoracotomy was used to expose the heart, and rhodamine (XRITC; Molecular Probes, Eugene, OR; 1% solution) and/or adenovirus that evokes expression of just GFP, or the $\epsilon$ subunit in addition to GFP, was topically injected into the pericardial sac on the surface of the cardiac tissue that contains the parasympathetic ganglia. The animals were allowed 2–4 days to recover. During the recovery period, XRTIC was transported retrogradely and thereafter could be used to identify the cell bodies of these cardiac parasympathetic neurons in the nucleus ambiguus. On the day of the experiment, the animals were reanesthetized with methoxyflurane and killed by rapid cervical dislocation. The hindbrain was quickly removed and placed in cold (0–4°C) buffer of the following composition: 140 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 5 mM glucose, 10 mM HEPES, and was continually gassed with 100% O$_2$. The medulla was then cut in transverse sections of 250-μm-thick slices using a vibratome. Slices were mounted in a perfusion chamber and submerged in a perfusate of the following composition: 125 mM NaCl, 3 mM KCl, 2 mM CaCl$_2$, 26 mM NaHCO$_3$, 5 mM dextrose, 5 mM HEPES, constantly bubbled with gas (95% O$_2$, 5% CO$_2$) and maintained at pH 7.4.

**Adenovirus Expressing the Human GABA$_{\alpha}$ $\epsilon$ Subunit**

The adenovirus was designed to express the human GABA$_{\alpha}$ $\epsilon$ subunit consisting of two cytomegalovirus promoters in head-to-tail orientation. The first promoter expresses the $\epsilon$ subunit and the second GFP. This results in expression of these proteins in the same cell but not to the same loci in each cell as these proteins are not fused. The adenovirus was made following the Adeasy principle, which allows for recombination of the shuttle vector with the rest of the adenoviral sequence in E. coli.

The full length sequence is then transfected into HEK 293 cells and amplified until a high titer stock is obtained. Titer was performed by flow cytometry quantifying the number of cells expressing GFP and is $7.74 \times 10^{10}$ transducing units per milliliter. This virus cannot replicate as the immediate early genes, E1 and E3, have been deleted and therefore cannot spread beyond the primary cardiac parasympathetic neurons. The second adenovirus was designed and made using identical techniques but including only the second cytomegaloviral promoter–GFP expression cassette.

**Patch Clamp Recording**

Patch pipettes were mounted onto a pipette holder and amplifier head stage (Axon Instruments Inc., Union City, CA; Axopatch 200B), which were connected to micromanipulators (Narashige International Inc., East Meadow, NY). The indifferent electrode was an Ag–AgCl plug submerged in the bath. Positive pressure was applied to the pipette as it was lowered into the bath to prevent clogging the tip with debris. The pipette was advanced until it began to form a seal with the cell membrane, which was seen as an increase in resistance. A brief period of suction created a gigaohm seal between the pipette and the cell membrane. Pipette capacitance was canceled at this stage. Once a gigaohm seal was formed, whole cell access to the inside of the cell was obtained by rupturing the membrane under the pipette tip. The bath solution was of the following composition: 120 mM NaCl, 4.8 mM KCl, 1.2 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, 5 mM HEPES, 5.5 mM dextrose, and 2 mM CaCl$_2$, equilibrated with 95% O$_2$–5% CO$_2$. Strychnine, D-2-amino-5-phosphonovalerate (50 μM), and CNQX (50 μM) were infused to prevent glycinergic, glutaminergic NMDA, and non-NMDA postsynaptic currents, respectively. The pipette solution was filled with the following composition: 150 mM KCl, 2 mM MgCl$_2$, 2 mM EGTA, 10 mM HEPES, 2 mM Mg-ATP, pH 7.4, and 5 mM QX314 was added to prevent sodium currents. With this pipette solution, the Cl$^-$ current induced by activation of GABA receptors was recorded as an inward current (calculated reversal potential of Cl$^-$: +4 mV). Postsynaptic currents were recorded in cardiac parasympathetic neurons at ~80 mV to avoid contamination from voltage-gated currents present at potentials less than ~50 mV (as shown in previous work).7,17,18

**Drug Application**

Pentobarbital (100–1,000 μm) was diluted with the perfusate solution immediately prior to use and was sequentially applied directly onto the neurons from tubing positioned 2.0 mm above the neuron under study. Drug solution was introduced using a pump at rate of 0.3 ml/min, and the solution was constantly suctioned out of the chamber at a fixed chamber height to keep the fluid within the recording chamber at a constant volume.
All drugs used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

Data and Statistical Analysis

Data were acquired using pCLAMP 8.0 software (Axon Instruments Inc.). Analysis of spontaneous synaptic currents was performed using MiniAnalysis (Synaptosoft, Decatur, GA; version 4.3.1) with an amplitude threshold of 8 pA. Averaged data are presented as mean ± SEM, n = number of cells. Responses were normalized to the maximum decay time, frequency, or amplitude recorded in that neuron. Statistical analysis was performed by using paired t tests to test for significance between two groups. P ≥ 0.05 was the criterion for statistical significance.

Results

The effect of pentobarbital on rhodamine identified parasympathetic cardiac neurons was determined by examining pentobarbital-evoked changes in the endogenous spontaneous GABAergic IPSCs in cardiac parasympathetic neurons. As shown in figure 1, pentobarbital increased the decay time but not the frequency or amplitude of the spontaneous IPSCs. As illustrated from a typical experiment, figure 1A, the duration of GABAergic IPSCs (average of 20) in control (left), pentobarbital (100 μM; middle), and recovery IPSCs (right) (A). The time course of IPSC decay (B), frequency (C), and amplitude (D) are shown for a representative experiment (left), and the average from experiments in nine cardiac parasympathetic neurons is shown (right). In this and all subsequent figures, the average data shown in the bar graphs (right) were normalized to maximal values of decay time, IPSC frequency, and amplitude from each neuron. In this and all subsequent figures, * denotes P ≥ 0.05.

Fig. 1. Pentobarbital augments IPSCs in parasympathetic cardiac neurons by increasing IPSC duration. Pentobarbital increased the duration of spontaneous IPSCs as shown by comparing the average of 20 IPSCs during control (left), pentobarbital (100 μM; middle), and recovery IPSCs (right) (A). The time course of IPSC decay (B), frequency (C), and amplitude (D) are shown for a representative experiment (left), and the average from experiments in nine cardiac parasympathetic neurons is shown (right). In this and all subsequent figures, * denotes P ≥ 0.05.

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and whether a second adenovirus that evokes expression of both GFP and the ε subunit of the GABA receptor could selectively transfect cardiac parasympathetic neurons. As shown in figure 2, top, the retrograde fluorescent tracer rhodamine can identify the cell bodies of cardiac parasympathetic neurons 2 days after the tracer was applied to the terminals of these neurons in cardiac ganglia at the base of the heart. Similar application of an adenovirus also successfully labeled these neurons by the expression of fluorescent GFP in the cell bodies of these neurons in the nucleus ambiguus (fig. 2, bottom).

In the neurons transfected with the second adenovirus that was engineered to coexpress both GFP and the ε subunit of the GABA receptor, pentobarbital had no effect on the spontaneous IPSCs (fig. 3). Figure 3A shows the average of 20 spontaneous GABAergic IPSCs before (left), during (middle), and after (right) application of 100 μM pentobarbital. As shown in a typical experiment in figure 3B (left) and in the summary data from seven neurons (right), the increase of decay time induced by pentobarbital (100 μM) was prevented by the transfection of GABA ε subunit receptor into cardiac parasympathetic neurons (fig. 3B). As expected, pentobarbital did not alter the frequency and amplitude of spontaneous GABAergic currents in these transfected cardiac parasympathetic neurons (figs. 3C and D).

To determine whether abolishing the pentobarbital sensitivity could be inadvertently caused by the adenovirus that evokes expression of GFP, rather than expression of the ε subunit of the GABA receptor, the effect of pentobarbital was examined in control experiments in which cardiac parasympathetic neurons were transfected with an adenovirus which expresses GFP but does not evoke expression of the ε subunit. In these cardiac parasympathetic neurons, the action of pentobarbital was preserved (fig. 4). Pentobarbital increased the duration of IPSCs from a control of 61 ± 7% to 80 ± 8% with 100 μM pentobarbital in cardiac parasympathetic neurons transfected with the adenovirus that does not express the GABA ε subunit (fig. 4A). As in cardiac parasympathetic neurons labeled only with the fluorescent rhodamine tracer, pentobarbital significantly increased the decay time but not the frequency or amplitude of the IPSCs. Figure 4A illustrates the spontaneous GABAergic recordings (average of 20 IPSCs) before (left), during (middle), and after (right) pentobarbital (100 μM) application. The time course of a typical experiment (left) and the average data from nine transfected cardiac parasympathetic neurons (right) are shown. Pentobarbital evoked an increase in decay time (fig. 4B), without any change in IPSC frequency (fig. 4C) or amplitude (fig. 4D). The absolute values of holding current, IPSC decay time, frequency, and amplitude from control cardiac vagal neurons, cardiac vagal neurons transfected with the ε subunit and GFP, and these neurons only transfected with GFP from a control period and during application of pentobarbital (100 μM) are shown in table 1.

**Discussion**

Although it is well accepted that the anesthetic pentobarbital blunts the baroreflex and increases heart rate, little is known about the mechanisms or responsible sites of action within the central nervous system. The present work provides two major findings that elucidate the action of pentobarbital on the specific neurons that are predominantly responsible for the control of heart rate. Pentobarbital, at concentrations of 100 and 1,000 μM, prolongs the duration of spontaneous IPSCs that impinge on cardiac parasympathetic neurons. These concentrations are clinically relevant as pentobarbital produces surgical anesthesia at concentrations of 200–300 μM. This action would augment the inhibition of cardiac parasympathetic neurons, thereby reducing parasympathetic cardioinhibitory activity and increasing heart rate. This work also demonstrates that expression of the GABA ε subunit in...
cardiac parasympathetic neurons, using an adenovirus, prevents the pentobarbital-evoked prolongation of spontaneous IPSCs in cardiac parasympathetic neurons transfected with the adenovirus that expresses the ε subunit of the GABA_A receptor as shown in a typical neuronal recording (A). The average of 20 IPSCs recorded under control conditions (left) were indistinguishable from the IPSCs recorded during application of pentobarbital (100 μM; middle) and during washout (right). The time course of IPSC decay time (B), their frequency (C), and amplitude (D) are shown for a representative experiment (left) and the average from experiments in seven cardiac parasympathetic neurons (right). IPSCs in cardiac parasympathetic neurons transfected with the adenovirus that expresses the ε subunit were rendered insensitive to pentobarbital (100 μM).

The severe (approximately 50%) inhibitory action of clinical doses of pentobarbital anesthesia on the reflex control of heart rate and blood pressure is well documented.2–4,6 Pentobarbital has minimal effects on the intrinsic rate of cardiac pacemaker cells in the sinoatrial node, indicating the site of action of pentobarbital is on parasympathetic neurons within the central nervous system.5 Although other cardiovascular neurons within the central nervous system may also be involved, this study demonstrates that one mechanism of action of pentobarbital is to increase the duration of spontaneous and endogenous inhibitory synaptic responses in parasympathetic cardiac neurons in the nucleus ambiguus. There are currently thought to be at least three major determinants of cardiac parasympathetic activity, including excitatory glutamatergic and nicotinic synaptic inputs, and an inhibitory GABAergic.

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neurotransmission to cardiac parasympathetic neurons.\textsuperscript{7} The increase in GABAergic inhibitory input with pentobarbital would reduce the activity of cardioinhibitory parasympathetic neurons, thereby increasing heart rate.

Nicoll \textit{et al.}\textsuperscript{21} first demonstrated that barbiturates, including pentobarbital, markedly prolong inhibitory postsynaptic potentials. This modulation occurs by increasing the mean open time of GABA-activated chloride channels.\textsuperscript{22} In this study, pentobarbital increased the duration but not the amplitude or frequency of spontaneous IPSCs in cardiac parasympathetic neurons. This is consistent with the work using other neurons that have also demonstrated pentobarbital does not alter the amplitude or time to peak of IPSCs but does increase the decay time.\textsuperscript{23}

The modulation of GABAergic function by anesthetics depends in part on the subunit composition of the GABA receptor.\textsuperscript{8,12,16} Expression of the \(\epsilon\) subunit alone does not produce functional GABA\(_\alpha\) or glycine receptors, indicating that this subunit cannot form homomeric ligand-gated Cl\(^-\) channels.\textsuperscript{12,24} However, recent work by Davies \textit{et al.}\textsuperscript{12} has demonstrated that expression of the \(\epsilon\) subunit in human embryonic kidney (HEK) cells renders the \(\alpha_1\beta_3\epsilon\) and \(\alpha_2\beta_1\epsilon\) GABA receptors insensitive to potentiation by barbiturates. However, unlike the results of Davies \textit{et al.}, \(\alpha_1\beta_1\epsilon\) subunits expressed in \textit{Xenopus} oocytes retained their ability to be facilitated by pentobarbital.\textsuperscript{24} Neclands \textit{et al.}\textsuperscript{16} also found \(\alpha_1\beta_1\epsilon\) as well as \(\alpha_1\beta_3\epsilon\) GABA receptors expressed in L929 cells were potentiated by pentobarbital. The reason for these conflicting results are unclear but could be due to different methods for examining potentiation with exogenous application of agonists. This is the first study to examine the effect of \(\epsilon\) subunit transfection on physiologically

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**Fig. 4.** Cardiac parasympathetic neurons infected with adenovirus that expresses GFP retained their sensitivity to pentobarbital. Pentobarbital increased the duration of spontaneous IPSCs as shown in A during control (left), pentobarbital (100 \(\mu\)M; middle), and washout (right) recordings from cardiac parasympathetic neurons transfected with adenovirus that expresses GFP. The time course of IPSC decay (B), their frequency (C), and amplitude (D) are shown for a representative experiment (left), and the average from experiments in eight cardiac parasympathetic neurons is shown (right). Similar to cardiac parasympathetic neurons labeled with rhodamine, in neurons transfected with the adenovirus that only expresses GFP, pentobarbital significantly increased the decay time of spontaneous GABAergic IPSCs in cardiac parasympathetic neurons (B) but did not change the IPSC frequency or amplitude (C and D, respectively).
important inhibitory GABAergic synaptic inputs in vitro. These results demonstrate prolongation of GABAergic postsynaptic currents by pentobarbital can be prevented by transfection of the ε subunit in cardiac vagal neurons, which is consistent with the hypothesis that the ε subunit abolishes potentiation by barbiturates.

Introduction of the ε subunit also has been shown to produce a spontaneous whole cell "leak" or holding current in the absence of an agonist.15,16 This current is likely caused by the increased spontaneous single-channel opening of GABA<sub>ε</sub> receptors containing the ε subunit.15,16 Although cardiac vagal neurons transfected with the ε subunit had a slightly larger holding current, this difference was not statistically significant (table 1, \( P = 0.2 \)).

The ε subunit may be distributed in brain regions that therefore have GABA<sub>ε</sub> receptors that are spared from the GABA-potentiation effect of general anesthetics. Several studies reveal that discrete brain regions and the electrical conduction system of the human heart contain the ε subunit mRNA.12,24 Northern analysis in human brain tissue indicates that the subunit is expressed in amygdala, thalamus, and subthalamic nucleus.12 In situ hybridization studies in squirrel monkey brain indicate that the hypothalamus and to a lesser extent the hippocampus express ε subunit mRNA.24 In rodents, ε subunit mRNA is found in the brain and heart, but the pattern of central nervous system distribution differs from that reported for primates.25,26 The rat locus ceruleus, brainstem, and hypothalamus contain a transcript that differs from the primate variety by virtue of its encoding approximately an extra 400 N-terminal amino acids.25 An alternatively processed ε subunit has been sequenced that shows greater amino acid identity with its presumed primate orthologue.26 According to in situ hybridization and immunohistochemical studies, this ε subunit is also located in the locus ceruleus, dorsal raphe, and the hypothalamus. The ε subunit mRNA is also present in the nucleus tractus solitarius of the rat, where it may be responsible for the reported benzodiazepine resistance of the expressed GABA<sub>ε</sub> receptors.27 However, there are currently no reports of the functional properties of recombinant rodent ε subunits. It remains to be determined whether they function like their human counterpart.

The physiologic role of the ε subunit is unknown. Interestingly, there is an increase in the expression of ε subunit mRNA in rodent dentate granule neurons relative to other GABA<sub>ε</sub> subunit transcripts following pilocarpine-induced seizures.28 Dentate granule neurons express ε subunit mRNA with that of multiple other subunit types, including the γ2 subunit, suggesting that these subunits may coexist in the same pentameric receptor complex. Recent recombinant studies reveal that α1, β3, γ2, and human ε subunits can exist in the same receptor and that these receptors can be distinguished from αβ, αβε, and αβγε receptors by virtue of their unique set of functional properties.13 Like αβε receptors, αβγε receptors have a diminished sensitivity to GABA-potentiation by anesthetic agents. GABA<sub>ε</sub> receptors native to cardiorespiratory neurons of the nucleus ambiguus may lack ε subunits, as evidenced by their sensitivity to modulation by pentobarbital. Introduction of recombinant ε subunit through adenoviral infection induced the abolition of modulation by pentobarbital. Whether this stems from the appearance of αβε and/or αβγε receptors remains to be determined.

Our work has shown that expression of the ε subunit into cardiac parasympathetic neurons renders the spontaneous GABAergic IPSCs insensitive to pentobarbital. This demonstrates that brainstem premotor neurons can be transfected with the GABA ε subunit in vitro using an adenovirus and, furthermore, that this transfection leads to insertion of the ε subunit into the native GABA<sub>ε</sub> receptors in these neurons and confers barbiturate insensitivity to the inhibitory synaptic response.

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Table 1. Pentobarbital Increases the Decay Time of GABAergic Inhibitory Postsynaptic Currents, Which Is Prevented by Transfection of the ε Subunit into Cardiac Vagal Neurons

<table>
<thead>
<tr>
<th></th>
<th>CVNs</th>
<th>CVNs Transfected with Both ε Subunit and GFP</th>
<th>CVNs Transfected Only with GFP</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control (100 μM)</td>
<td>Pentobarbital</td>
<td></td>
</tr>
<tr>
<td>Holding current (pA)</td>
<td>−280 ± 44</td>
<td>−313 ± 60</td>
<td>−301 ± 28</td>
</tr>
<tr>
<td></td>
<td>182 ± 2</td>
<td>339 ± 10*</td>
<td>179 ± 1</td>
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<tr>
<td>IPSC decay time (ms)</td>
<td>17.2 ± 5.4</td>
<td>18.0 ± 6.6</td>
<td>18.8 ± 5.2</td>
</tr>
<tr>
<td></td>
<td>−187.6 ± 13.4</td>
<td>−196.3 ± 1.3</td>
<td>−186.6 ± 0.8</td>
</tr>
</tbody>
</table>

Pentobarbital did not significantly alter the holding current, frequency, or amplitude of GABAergic inhibitory postsynaptic currents (IPSCs) in any of the three groups of cardiac vagal neurons. The only effect of pentobarbital was to significantly increase the decay time of GABAergic IPSCs in cardiac vagal neurons (n = 9). The control holding current, IPSC frequency, and amplitude in cardiac vagal neurons transfected with the ε subunit (n = 7) were not significantly different from nontransfected cardiac vagal neurons (n = 8). However, in cardiac vagal neurons transfected with the ε subunit, pentobarbital failed to significantly alter the IPSC decay time. Cardiac vagal neurons transfected with the adenovirus, which only expresses green fluorescent protein, responded similarly to control cardiac vagal neurons, and pentobarbital did not significantly alter the holding current, frequency, or amplitude of GABAergic IPSCs but did increase their decay time.

* \( P < 0.05 \).

CVN = cardiac vagal neurons; GFP = green fluorescent protein; IPSC = inhibitory postsynaptic currents.
References

17. Mihalevich M, Neff RA, Mendelowitz D: Voltage-gated currents in identified parasympathetic cardiac neurons in the nucleus ambiguus. Brain Res 1996; 749:258–62

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