A COMBINED BLOCKADE OF GLYCINE AND CALCIUM-DEPENDENT POTASSIUM CHANNELS ABOLISHES THE RESPIRATORY RHYTHM

D. BÜSSELBERG,* A. M. BISCHOFF AND D. W. RICHTER

Georg-August-Universität Göttingen, Abteilung für Neuro- und Sinnesphysiologie, Humboldtallee 23, D-37073 Göttingen, Germany

Abstract—In order to test whether glycinergic inhibition is essential for the in vivo respiratory rhythm, we analysed the discharge properties of neurones in the medullary respiratory network after blockade of glycine receptors in the in situ perfused brainstem preparation of mature wild type and oscillator mice with a deficient glycine receptor.

In wild type mice, selective blockade of glycine receptors with low concentrations of strychnine (0.03–0.3 μM) provoked considerable changes in neuronal discharge characteristics: The cycle phase relationship of inspiratory, postinspiratory and expiratory specific patterns of membrane potential changes was altered profoundly. Inspiratory, postinspiratory and expiratory neurones developed a propensity for fast voltage oscillations that were accompanied by multiple burst discharges. These burst discharges were followed by “after-burst” hyperpolarisations that were capable of triggering secondary burst discharges. Blockade of glycine receptors and the “big” Ca2+-dependent K+-conductance by charybdotoxin (3.3 nM) resulted in loss of the respiratory rhythm, whilst only tonic discharge activity remained. In contrast, rhythmic activity was only weakened, but preserved after the “small” Ca2+-dependent activated K+-conductance was blocked with apamin (8 nM). Also low concentrations of pentobarbital sodium (6 mg/kg) abolished rhythmic respiratory activity after blockade of glycine receptors in the wild type mice and in glycine receptor deficient oscillator mice.

The data imply that failure of glycine receptors provokes enhanced bursting behaviour of respiratory neurones, whilst the additional blockade of BKCa channels by charybdotoxin or with pentobarbital abolishes the respiratory rhythm. © 2003 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: rhythm generation, synaptic interactions, network functions, breathing behaviour, mice, oscillator mice.

Under in vivo conditions, periodic synaptic inhibition through glycinergic and GABAAergic synapses is believed to exert a principal function in the respiratory network (Richter, 1996; Champagnat et al., 1982; Haji et al., 1992; Schmid et al., 1996; Shao and Feldman, 1997). The functional significance of glycinergic inhibition for respiratory rhythm generation became obvious when microinjections of the glycine receptor blocker strychnine into the pre-Bötzinger complex of anaesthetized mature cats resulted in an absolute cessation of rhythmic respiratory discharges (Pierrefiche et al., 1998). A recent study, however, showed that the respiratory rhythm persists in the non-anaesthetized brainstem preparation in wild type mice after failure of glycinergic inhibition (Büsselberg et al., 2001a) as well as in mutant oscillator mice (Büsselberg et al., 2001b). This observation was unexpected, since these oscillator mice were shown to have a complete loss of the mature α1, β type glycine receptors (Kling et al., 1997), whilst the embryonic α2 and the α3, β receptor subtypes were supposed to be absent at the age these animals were used at postnatal age of P18 to P22. Persistence of the respiratory rhythm after failure of glycinergic inhibition raised the question whether membrane properties of neurones may play a key role in the in vivo respiratory rhythm generation of non-anaesthetized mammals (Richter, 1996).

To further investigate the significance of glycinergic inhibition for respiratory rhythm generation in an in vivo like condition, we have analysed cellular and network responses after selective blockade of glycine receptors in wild type mice (C57BL/6J or NMRI) as well as in homocysteic oscillator mice (spd/spd) in the in situ perfused brain stem preparation (Paton, 1996). This preparation allows analysis of specific synaptic processes and testing for the functional role of membrane properties in an intact respiratory network under non-anaesthetized conditions. Our studies show that respiratory neurones develop spontaneous voltage oscillations and secondary bursts when glycinergic inhibition fails and suggest that a “big” Ca2+-dependent K+-conductance (BKCa) conductance is essential for the maintenance of respiratory rhythm. A clinically relevant finding was that low doses of pentobarbital abolish rhythmic respiratory discharges after failure of glycine receptors as it may occur in patients with hyperekplexia.

A preliminary report has been published in abstract form (Büsselberg et al., 2000).

EXPERIMENTAL PROCEDURES

Surgical procedures and preparation

Analyses were performed on the in situ perfused brainstem preparation (Paton, 1996) of wild-type (C57BL/6J or NMRI; postnatal age P20–P25) and mutant oscillator (spd/spd; postnatal age P18–23) mice, which were bred and housed in the University of Göttingen (Göttingen, Germany) animal facility. Oscillator mice were identified by their phenotype and by PCR analysis of tail tissue.

All experimental procedures conformed to the recommendations of the European Commission (No L 358, ISSN 0378-6978),...
and protocols were approved by the Committee on Animal Research of the University of Göttingen. Every attempt was made to minimize the number of animals used in this study. Our procedures minimized pain and discomfort of animals used. Furthermore mice were deeply anaesthetized with halothane as verified by the lack of nociceptive reactions to pinches applied to the paws and the tail. During maintained anesthesia, brains were transected at the pre-collicular level and the rostral part to the transection as well as the cerebellum was removed by suction. Following a second transection through the thorax below the level of the diaphragm, the thorax–brainstem preparation was isolated and placed in an ice-chilled bath of artificial cerebrospinal fluid (ACSF) that contained: 125 mM NaCl, 24 mM NaHCO₃, 5 mM KCl, 2.5 mM CaCl₂, 1.25 mM MgSO₄, 1.25 mM KH₂PO₄, 1.1 mM glucose. The ACSF had an osmolarity of 290 mosmol and was gassed with 95% O₂/5% CO₂ (pH was adjusted to 7.4 with HCl or NaOH). The preparation was transferred to a recording chamber that contained: 2.1 g dextran/100 ml (total osmolarity of 300–320 mosmol), glucose. The ACSF had an osmolarity of 290 mosmol and was gassed with 95% O₂/5% CO₂ (pH was adjusted to 7.4 with HCl or NaOH). The preparation was transferred to a recording chamber where the descending abdominal aorta was quickly (within 7–10 min) cannulated for retrograde perfusion with ACSF containing 2.1 g dextran/100 ml (total osmolarity of 300–320 mosmol), gassed with either 8% (ACSF pH 7.3) or 12% CO₂ (ACSF pH 7.2) in oxygen and maintained at a temperature of 31 °C. The perfusate was driven by a peristaltic pump and re-circulated through filters and bubble traps. The perfusion pressure was measured continuously by a transducer at the level of the descending abdominal aorta and maintained at 50–60 mm Hg by adjusting the flow rate. Vetricum bromide 0.04 μg ml⁻¹ (Norcuron, Organon Teknika, Germany) was added to the perfusate to maintain muscle paralysis.

Phrenic nerves (PN) were prepared for recordings by a dorsal approach.

Electrophysiological analysis

Neural discharges from the PN were recorded with glass suction electrodes to monitor central respiratory activities. The neural activities were amplified (2000–10,000×), band-pass filtered (100–5000 Hz), monitored on an oscilloscope (Tektronix, Beaverton, OR, USA) and registered as raw discharge activity or in integrated form (τ=0.1 s) on a strip chart recorder (Yokogawa, Japan).

Intracellular recordings from medullary respiratory neurones were obtained with an npi amplifier (Tamm, Germany) using fine-tipped glass micropipettes (tip size approximately 0.5 μm) filled with 2 M potassium methylsulfate (ICN Biomedicals GmbH, Germany) and 5 mM 1,2-bis[2-aminophenoxy]ethane-N,N',N''-N',tetraacetic acid (Sigma-Aldrich Chemie GmbH, Germany). Serial resistances as measured in extracellular fluid ranged between 40 and 80 MΩ. Respiratory neurones were recorded in the ventral respiratory group of the medulla oblongata and functionally identified by the periodic fluctuations of their membrane potential and their discharge in synchrony with PN activity. According to the fair ventral position of recording sites, we have presumably excluded cranial motoneurones. The membrane potential was monitored on-line on an oscilloscope and on a strip chart recorder.

Drug solutions

Drugs were applied to the perfusate solution. Strychnine hydrochloride (Sigma, Taufkirchen, Germany), a selective glycine receptor antagonist, was applied in 0.03–0.3 μM concentrations to ensure receptor specificity (Jonas et al., 1998). The “small-conductance” SKCa channel blocker apamin (Sigma) was applied in a concentration of 8 nM (Pennefather et al., 1985; Hill et al., 1992) and the “big-conductance” BKCa channel blocker charybdotoxin (CTX, Alomone, Jerusalem, Israel) was applied in a concentration of 3.3 nM (Egan et al., 1993). Both drugs were dissolved in ACSF with bovine serum albumin (Sigma), 0.03 g/100 ml instead of dextran. Control solutions containing bovine serum albumin with-
i.e. inspiratory (N=39), post-inspiratory (N=10) and expi-
tratory (N=14) neurones after glycine receptor blockade,
whilst respiratory rhythm generation was preserved.

Changes in inspiratory neurones
Under control conditions, inspiratory neurones exhibited
an augmenting pattern of membrane depolarisation and

Fig. 1. Characteristic changes of burst discharges in PN of wild type mice under control conditions and after blockade of glycine receptors with
strychnine: A) Prolongation of inspiratory bursts and secondary bursts after application of strychnine. B) Double or triple bursts occur when the glycine
receptor is blocked. Traces on the right panel demonstrate the integrated PN activity. C) Fast voltage oscillations during the post-inspiratory period
after application of strychnine.

PN discharges in relation to changes of membrane voltages in an inspiratory (A) and an expiratory (B) neuron. A) Double and triple bursts occur
after the application of strychnine. B) Burst discharges in an augmenting expiratory neuron after glycine receptor blockade. Control, showing maximal
synaptic inhibition during early inspiration and a slowly augmenting discharge of action potentials with small after-hyperpolarisations during the
expiratory interval. During glycine receptor blockade with strychnine (0.3 μM), PN was silent for a short period of time during inspiration (arrow). ECG,
electrocardiographic activity; PN, PN discharge. PN and ECG are recorded simultaneously.
action potential discharges which lasted throughout the presence of strychnine, several notable changes in the spontaneous variations of the membrane potential and action potential discharge occurred consistently. (i) Inspiratory membrane depolarisation became significantly steeper, whilst the duration from the resting membrane potential to the peak of depolarisation decreased from 0.5 ± 0.12 s to 0.27 ± 0.04 s (P-value = 0.01), the amplitude of the depolarisation increased from 17.1 ± 1.2 mV to 20.1 ± 1.4 mV (P-value < 0.01). The action potential discharges became more intense and changed from 10.7 ± 2.9 action potentials per bursts to 26.3 ± 4.3 action potentials per burst (P-value < 0.01) whilst the frequency increased from 32.2 ± 7.8–58.4 ± 8.1 action potentials per second (P-value < 0.01). (ii) The duration of the membrane depolarisation was reduced to roughly half compared with control (from 0.92 ± 0.4 s to 0.54 ± 0.2 s; N=5; P-value = 0.06). (iii) The steepness of membrane repolarisation following inspiratory bursts was slowed and the voltage hyperpolarisation normally occurring during post-inspiration disappeared. (iv) Clustered waves of depolarizing potentials occurred at 4–6 Hz (Fig. 4A) in association with doublet bursts in PN output activity (Figs. 2A; 3A, B; 4A) and sometimes (N=4) even in the absence of PN discharges (Fig. 4B). Strychnine blockade of glycine receptors enhanced inspiratory depolarisations in most inspiratory neurones (N=30 of 39; Fig. 3A, B). The strongly augmented inspiratory depolarisation was followed by a pronounced after-burst hyperpolarisation, their amplitudes changing from 1.6 ± 0.1 mV under control conditions to 16.5 ± 5.4 mV after the application of strychnine (P-value < 0.01), whilst the after-burst hyperpolarisation after extra-bursts was 21.5 ± 6.3 mV (P-value compared with the strychnine data: 0.08; N=8). These enhanced after-burst hyperpolarisations brought the membrane potential below pre-inspiratory, i.e. even below inhibitory synaptic levels (Fig. 3A) and were frequently followed by an interposed "doublet-burst" within the same cycle phase, i.e. without

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**Fig. 3.** Blockade of glycineric inhibition revealed double bursting. A/B) Potentiation of burst-related membrane depolarisations and prominent after-burst hyperpolarisation that were followed by extra-burst (arrows) depolarisations in two different inspiratory neurones. Upper traces: membrane potentials; ECG, electrocardiographic activity; PN: PN activity.
preceding expiratory interval (Fig. 3B, arrow). Under control conditions, inspiratory neurones \((N=9\) of 39) discharged only a short series of fast action potentials at the very onset of inspiratory depolarisation (Fig. 3B). After strychnine application, the neurons changed this “adaptive” firing pattern and discharged action potentials even throughout part of the expiratory interval (Fig. 3B, right side) until they were finally stopped shortly before onset of the next inspiratory phase. In several inspiratory neurones \((N=5\) from 39), fast sub-threshold oscillations of membrane depolarisations appeared repeatedly after the end of inspiratory bursts and continued into the expiratory intervals. This was accompanied by 4–6 Hz oscillations in PN discharges (Fig. 4A).

**Fig. 4.** Fast oscillations occur in membrane voltage as well as in PN activity in different types of respiratory neurones. A) Blockade of post-inspiratory membrane potential hyperpolarisation and fast voltage oscillations during the post-inspiratory period in an inspiratory neuron. B) An inspiratory neuron with repetitive depolarization waves during the post-inspiratory period that were accompanied by brief discharges of PN activity. (Note different time scales!) C) During strychnine application a post-inspiratory neuron started to depolarise during inspiration and rapid voltage oscillations in synchrony with PN activity occurred during the inter-burst interval.

**Changes in expiratory neurones**

During control, augmenting expiratory neurones were hyperpolarised to maximal voltages during early inspiration, and significant synaptic inhibition outlasted inspiration into post-inspiration. Therefore, expiratory neurones started to discharge only gradually after end of postinspiration. Termination of membrane depolarisation and expiratory discharge occurred rapidly during late-expiration and onset of
PN discharges (Fig. 2B). After blockade of glycine receptors, the voltage fluctuations attained a step-like pattern, because post-inspiratory inhibition was absent, whilst synaptic hyperpolarisation persisted during the inspiratory phase (Fig. 2B). Previously augmenting expiratory neurones therefore depolarised rapidly from strongly hyperpolarised inspiratory levels to maximal expiratory depolarisation. The duration of expiratory depolarisation did not change (2.1±0.8 s versus 2.1±1.1 s; N=6) after application of strychnine. An important observation made in several cases (N=3) was that inspiratory inhibition did not reveal clear fragmentations, although strychnine blockade of glycine receptor had induced doublet bursting in PN discharge (Fig. 2B).

Changes in post-inspiratory neurones

Blockade of glycine receptors also dramatically changed the discharge of post-inspiratory neurones. Under control conditions, post-inspiratory neurones were hyperpolarised to maximal voltage levels at the beginning of the inspiratory phase (Fig. 4C). After blockade of glycine receptors, (i) membrane depolarisation and onset of action potential discharges were shifted into the inspiratory phase (see Büsselberg et al., 2001a). (ii) The periodic discharge was intensified from 35±12 action potentials per depolarisation to 87±14 action potentials per depolarisation (P-value<0.01) and the membrane depolarisation was prolonged into the inspiratory phase. (iii) In four (out of 10) neurones the slowly decaying “post-inspiratory” burst discharges were greatly altered. The initial post-inspiratory burst discharges were intensified and followed by a secondary burst-depolarisation. At the same time, PN activity was depressed (not shown).

Another characteristic feature that appeared after glycine receptor blockade in post-inspiratory neurones (N=8 of 10) were rapid voltage oscillations combined with action potential discharges in synchrony with high-frequency PN discharges (Fig. 4C). In such cases, the silent period characteristic of the expiratory interval was missing in PN activity.

Glycine receptor blockade abolished the respiratory rhythm in pentobarbital-anaesthetized and mutant oscillator mice

The data described above are in apparent conflict with a previous study, which demonstrated that in the pentobarbital-anaesthetized cat, strychnine injected into the pre-Bötzinger complex blocked rhythmic respiratory activity (Pierrefiche et al., 1998). Since a major difference in the experimental approach of the two studies was anaesthesia, we tested the effect of pentobarbital after glycine receptor blockade in post-inspiratory neurones (N=8 of 10) were rapid voltage oscillations combined with action potential discharges in synchrony with high-frequency PN discharges (Fig. 4C). In such cases, the silent period characteristic of the expiratory interval was missing in PN activity.

Fig. 5. Pentobarbital induced central apnoea when glycine receptor is absent. A) Action of pentobarbital and strychnine in wild type mice. Upper trace: PN discharges before treatment. Middle trace: PN discharges were reduced after administration of pentobarbital sodium (Nembutal; 6 mg/kg); Lower trace: PN discharges were blocked by additional application of strychnine, 0.3 μM. B) Pentobarbital applied in oscillator mice induces apnoea.
of drug application was reversed (N=3), strychnine being applied before pentobarbital. Such findings revealed a depressant action of pentobarbital on the bursting mechanisms of neurones and resolved the discrepancy between the two studies. Administration of the same small dose of pentobarbital to oscillator mice (spd<sup>−/+</sup> spd<sup>−/−</sup>, N=3) also resulted in a loss of rhythmic PN activity within short times (Fig. 5B).
Disruption of the respiratory rhythm after blockade of calcium-activated potassium channels

To test the possibility that after-burst hyperpolarisations preceding doublet bursting after glycine receptor blockade were linked to activation of Ca\(^{2+}\)-dependent K\(^+\) currents, we examined the effects of blocking the small-conductance of Ca\(^{2+}\)-dependent K\(^+\) channels (SK\(\text{Ca}\)) with apamin and the BK\(\text{Ca}\) with CTX.

Apamin administration \((N=7)\) after strychnine blockade of glycine receptors in wild type mice did not abolish respiratory activity (Fig. 6). The intensity of both PN burst discharges and cellular discharges gradually declined and became obvious after 15–45 min, but there was no obvious change in burst characteristics (Fig. 6). The shape of single action potentials and after-hyperpolarisations also remained unchanged (not illustrated).

Strikingly different was the response to blockade of BK\(\text{Ca}\) by CTX \((3.3 \text{nM})\). The changes progressed with time until all rhythmic burst discharges disappeared, whilst enhanced tonic discharges persisted (Fig. 7E, I).

**DISCUSSION**

The "Conditional Network-Burster Theory" (Richter et al., 1986, 2000; Richter, 1996; Richter and Spyer, 2001) postulates that synaptic inhibition via glycinergic and/or GABAergic synapses (Champagnat et al., 1982; Haji et al., 1992, 2000; Schmid et al., 1996) is important for normal respiratory rhythm generation in the in vivo mature mammal (Pierrefiche et al., 1998). Since it is known,
that barbiturates suppress respiratory rhythm (Robson et al., 1963), we used the non-anaesthetized in situ perfused brainstem preparations (Paton, 1996) to specifically test the contribution of glycineric inhibition by using low concentrations of strychnine (in the range of 0.03–0.3 μM) in order not to affect at the same time GABAA receptors (Jonas et al., 1998) and provoke epileptiform activities as verified by the absence of drug-induced discharges in non-respiratory nerves of the brachial plexus (Büsselberg et al., 2001a). The study revealed that failure of glycineric inhibition induces multiple forms of rhythmic discharges and voltage oscillations in all three types of medullary respiratory neurones. Whilst these changes of firing behaviour could also be explained by changes in the efficacy of synaptic interactions, our interpretation is that medullary respiratory neurones developed an intrinsic tendency to cellular bursting when they are released from inhibitory synaptic control through glycine receptors. We speculated therefore that Ca2+-dependent K+ currents, activated by preceding Ca2+ currents (Mironov and Richter, 1998; Elsen and Ramirez, 1998; Pierrefiche et al., 1999), are involved in termination of rhythmic bursting. A consequence therefore should be loss of rhythmic respiratory bursting, when "big" conductance BKCa channels were blocked with CTX. This was indeed the case; rhythmic respiratory activity disappeared prevailing irregular tonic discharges. Therefore we suggest that the mature in vivo respiratory network is capable of changing between two operational modes: Synaptic inhibitory interaction between respiratory neurones seems to be important for respiratory rhythm generation and stabilisation under normal conditions (Richter et al., 1986; Richter and Spyer, 2001). However, whenever glycineric inhibition is diminished, mature respiratory neurones may enter a different operational state in which intrinsic bursting becomes increasingly important, as is described for neonatal neurones (Smith et al., 1991; Ramirez and Richter, 1996).

**Persistence of respiratory rhythm generation after glycine receptor blockade**

A series of previous studies (Pierrefiche et al., 1998; Büsselberg et al., 2001a) revealed that inspiratory, post-inspiratory and stage two expiratory neurones receive glycineric inhibition during the inspiratory phase. In addition, there is glycineric inhibition during the post-inspiratory phase in inspiratory and stage two expiratory neurones (Champagnat et al., 1982; Schmid et al., 1996; Haji et al., 1992). The importance of glycineric inhibition became evident when microinjections of strychnine into the pre-Bötzinger complex of anaesthetized cats eliminated rhythmic respiratory activity (Pierrefiche et al., 1998). The most reasonable explanation was that such neural apnoea resulted from removal of glycineric synaptic interactions between the different types of respiratory neurones in the pre-Bötzinger complex (Connelly et al., 1992; Schwarzacher et al., 1995). Conversely however, respiratory rhythm persisted in non-anaesthetized mature mutant oscillator mice (Büsselberg et al., 2001b) that lack functional glycine receptors (Kling et al., 1997) and in the in vitro neonatal brainstem after blockade of glycine receptors (Onimaru et al., 1987, 1989; Feldman et al., 1990). There are two possible explanations for these differences: (i) respiratory neurones have the potential for endogenous bursting, which is normally overwritten by inhibitory synaptic control and concomitant conductance changes (Richter and Spyer, 2001); (ii) endogenous bursting depends on specific membrane conductances, which are suppressed by barbiturates. Such barbiturate sensitivity was indeed verified for fast Na+ channels (Rehberg and Duch, 1999), rectifying K+ channels (Gibbons et al., 1996), pre- and postsynaptic high voltage-activated Ca2+ channels (Werz and Macdonald, 1985), the BKCa channels (Ishida et al., 1999) as well as GABAA receptor currents (Saunders and Ho, 1990). We assume that this explains why endogenous respiratory bursting is attenuated in anaesthetized preparations.

**Mechanisms of double bursts and fast oscillations**

Several changes in inspiratory activity developed when glycineric synapses were blocked: (i) respiratory movements producing long-lasting bursts were doubled; (ii) membrane hyperpolarisation of inspiratory neurones starting with onset of the post-inspiratory phase was slowed; and (iii) all types of neurones developed a preference for voltage oscillations at a frequency of 4–6 Hz. All these changes are consistent with the view that glycineric inhibition is required for effective late- and post-inspiratory “off-switching” of respiratory phase activities (Richter et al., 1979; Richter, 1982; Schmid et al., 1996).

The various types of respiratory neurones tested developed a tendency to generate multiple burst discharges, which often were accompanied by corresponding changes in PN output activity. The responses of individual neurones resembled the general pattern described for endogenous bursting of respiratory neurones in the in vitro slice preparation (Johnson et al., 1994). In our study, “additional” bursts were initiated by a pronounced membrane hyperpolarisation following enhanced preceding bursts. We assume that such post-burst membrane hyperpolarisation originated from KCa currents (Mifflin et al., 1985; Richter et al., 1993; Pierrefiche et al., 1995), which obviously are competent to re-activate inactivating voltage-regulated membrane conductances to trigger a secondary burst. The obvious test therefore was to specifically block BKCa channels. Such blockade abolished all rhythmic discharges, inspiratory bursts becoming longer and smaller, whilst tonic discharge activity persisted. Therefore, activation of BKCa channels seems to be essential for maintenance of repetitive firing during bursts and burst termination in glycine receptor-deficient mice (Mironov, 1983; Bennett et al., 2000; Shao et al., 1999; Golding et al., 1999; Pedarzani et al., 2000).

**Therapeutic implications**

Under mature in vivo conditions, periodic breathing requires not only a persistent excitatory synaptic drive, but also mutual inhibitory control by glycineric and GABAergic synaptic inhibition. Our findings suggest that endoge-
nous bursting behaviour of respiratory neurones are sup-
pressed by synaptic inhibition, but might be released,
whenever inhibitory synaptic control declines or is sup-
pressed by pathologic processes. Patients suffering from
hyperekplexia lack functional glycine receptors. In these
patients, respiratory rhythm might depend on a certain
endogenous bursting capacity of respiratory neurones.
The high sensitivity of these bursting mechanisms to pen-
tobarbital calls for careful monitoring and cautious admin-
istration of barbiturates when they are necessary in the
treatment of hyperekplexic patients.

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