BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1998 Jul; 19 (4): 327 - 331

Dual effects of pentobarbital on rat sacral dorsal commissural neurons *in vitro*[1]

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KEY WORDS pentobarbital; GABA; patch-clamp techniques; neurons; spinal cord

AIM: To study the effects of pentobarbital (PB) on acutely dissociated rat sacral dorsal commissural neurons (SDCN). **METHODS:** Nystatin-perforated patch clamp recording was used. **RESULTS:** (1) At a holding potential of - 40 mV, PB induced inward Cl^- current (I_{PB}) in a concentration-dependent manner with a EC_{50} (95 % confidence limits) of 416 (385 - 477) $\mu mol \cdot L^{-1}$ and a Hill coefficient of 1.08. (2) Picrotoxin reversibly blocked I_{PB}. (3) The reversal potential of I_{PB} was close to the Cl^- equilibrium potential. (4) PB enhanced GABA-induced Cl^- influx (I_{GABA}). In the presence of PB 30 $\mu mol \cdot L^{-1}$, the EC_{50} (95 % confidence limits) of I_{GABA} decreased from 6.9 (5.4 - 8.4) $\mu mol \cdot L^{-1}$ to 3.5 (2.9 - 4.1) $\mu mol \cdot L^{-1}$. **CONCLUSION:** PB had dual effects on SDCN, facilitated $GABA_A$ receptor-mediated currents, and at higher concentrations induced Cl^- influx itself.

Anesthetics-induced suppression of pain-evoked movement was mediated at the spinal cord level, rather than in supraspinal sites[1]. In both intact and spinal rats, pentobarbital (PB), prevented the development of C-fiber-induced hyperalgesia[2], suggesting that at least a part of anesthetics-induced depression of nociceptive response is due to blockade of such responses in spinal dorsal horn neurons. In the dorsal root ganglion cells of frog[3] and in the dissociated cerebellar Purkinje cells, nucleus tractus solitarius, and suprachiasmatic nucleus neurons of rat[4-6], PB augments the Cl^- conductance evoked by GABA, and PB itself also induce Cl^- current. In order to elucidate the actions of the general anesthetic within the spinal cord, we studied the effects of PB on acutely dissociated rat sacral dorsal commissural neurons (SDCN), which have been implicated in relaying nociceptive information[7-9].

MATERIALS AND METHODS

Preparation of SDCN Two-week-old Sprague-Dawley rats (Certificate to the rats No 08-014 Shaanxi; Certificate to the animal house No CTJ95002, PLA) were decapitated under pentobarbital sodium anesthesia. Spinal cord ($L_5 - S_2$) was sectioned with a vibratome (DTK-1000, Dosaka) to slices 400 μm thick containing the SDCN region. The slices were preincubated in oxygenated standard external solution (SES) at 22 - 25 ℃ for 50 min. Then the slices were treated with SES containing pronase 0.125 - 0.167 $g \cdot L^{-1}$ at 31 ℃ for 20 min, followed by exposure to thermolysin 0.125 - 0.167 $g \cdot L^{-1}$ for another 15 min. The slices were kept in enzyme-free SES for 1 h. The SDCN region was micro-punched out with an electrolytically polished injection needle and transferred into a culture dish (Falcon) filled with SES. Neurons were mechanically dissociated with fire-polished Pasteur pipettes under a phase-contrast microscope (Olympus, IX70). Dissociated neurons, which adhered to the bottom of the dish within 20 min, were used for the electrophysiological studies.

Solutions The SES contained NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, and glucose 10 $mmol \cdot L^{-1}$; pH was adjusted to 7.4 with Tris-base. The pipette solution contained KCl 150 and HEPES 10 $mmol \cdot L^{-1}$; pH was adjusted to 7.2 with Tris-base. A nystatin stock solution in acidified methanol at a concentration of 10 $g \cdot L^{-1}$ was prepared and stored at - 20 ℃. The stock solution was added to the pipette solution with nystatin at a final concentration of 200 $mg \cdot L^{-1}$ just before use.

[1] Project supported by the National Natural Science Foundation of China. No 39770248.
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Received 1997-08-11 Accepted 1997-12-16

When the current-voltage ($I - V$) relationship for the PB-induced current was examined, tetrodotoxin (TTX) 0.3 $\mu mol \cdot L^{-1}$ and $CdCl_2$ 10 $\mu mol \cdot L^{-1}$ were added to the external solution; CsCl 150 $mmol \cdot L^{-1}$ was substituted for KCl in the pipette solution.

Electric measurement Electric measurement was done using nystatin-perforated patch recording configuration under voltage-clamp condition at 22 – 25 ℃. Patch pipettes were pulled from glass capillaries (OD 1.5 mm) on a two-stage puller (PB-7, Narishige). The resistance between the recording electrode filled with pipette solution and the reference electrode was 4 – 6 MΩ. The electrode was connected to a patch-clamp amplifier (CEZ-2300, Nihon Kohden), filtered at 1 kHz. The current and voltage signals were monitored with a pen recorder (Omniace RT 3108, San-ei), sampled and analyzed using a DigiData 1200A interface and a computer (Compaq Presario 7170) with pCLAMP 6.0.2 software (Axon Instruments). The membrane potential was held at – 40 mV, except when $I - V$ relationship was examined. Measurements were started after the stabilization of the GABA response (15 – 20 min after cell attachment mode was obtained).

Drugs Pronase (Calbiochem), thermolysin, nystatin, picrotoxin, TTX, bicuculline (Sigma), and pentobarbital sodium (Union), GABA (Tokyo Kasei) were used. Rapid medication was given with the "Y-tube" method[8,9], which allowed a complete exchange of external solution surrounding a neuron within 20 ms.

Statistical analysis Data were presented as $\bar{x} \pm s$. EC_{50} and Hill coefficient were assessed from the Michaelis-Menten equation using a least-squares method:

$$I = I_{max} \cdot C^n / (C^n + EC_{50}^n),$$

where I was current, I_{max} was the maximal response, n was the Hill coefficient, EC_{50} was the half-maximal effective concentration, and C was the concentration of agonists. All currents were normalized to GABA 10 $\mu mol \cdot L^{-1}$ when the concentration-response curve was plotted and each point was the mean of 6 records. When the $I - V$ relationship was examined, all records were normalized to the currents induced at a holding potential of – 40 mV and each point represented the mean of 4 records.

RESULTS

PB-induced inward current Application of PB evoked inward current (I_{PB}) in all SDCN tested at a holding potential of – 40 mV. The current became detectable at a concentration of 100 $\mu mol \cdot L^{-1}$ and increased in a concentration-dependent manner (Fig 1Aa), leading to a sigmoid-shaped concentration-response curve with a EC_{50} (95 % confidence limits) of 416 (385 – 447) $\mu mol \cdot L^{-1}$ and a Hill coefficient of 1.08 (Fig 1B). The wash-out of PB at concentrations higher than 3 $mmol \cdot L^{-1}$ always induced a transient tail inward current (hump current) (Fig 1Aa). The I_{PB} was reversibly blocked by picrotoxin, that blocked the Cl^- channels coupling to $GABA_A$ receptors (Fig 1Ab). In the presence of picrotoxin 100 $\mu mol \cdot L^{-1}$, the

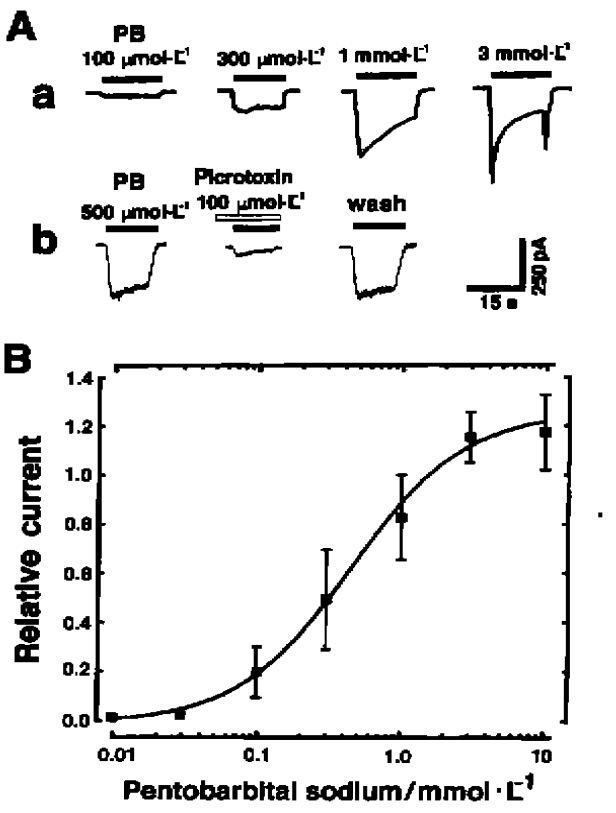

Fig 1. PB-induced inward currents in SDCN. Aa: the inward currents induced by different concentrations of PB at a holding potential of – 40 mV. Ab: the current responses to PB 500 $\mu mol \cdot L^{-1}$ alone (left), in the presence of picrotoxin 100 $\mu mol \cdot L^{-1}$ (middle), and alone again (right). B: the concentration-response curve of PB-induced currents.

current induced by PB 500 μmol·L^{-1} decreased by 95 % ± 3 % (n = 4). The concentration-response relationship of I_{PB} peak current component was summarized in Fig 1B.

Current-voltage ($I-V$) relationship for I_{PB} To determine the ion responsible for I_{PB} in SDCN, the $I-V$ relationship for I_{PB} was examined. Fig 2A showed the currents induced by PB 500 μmol·L^{-1} at various holding potentials. The reversal potential (E_{PB}) of I_{PB} was 0.66 ± 0.08 mV (n = 5, Fig 2B), which was close enough to the theoretical Cl^- equilibrium potential (E_{Cl}) of − 1.8 mV calculated with the Nernst Equation from the given extra- and intracellular Cl^- activities (161 and 150 mmol·L^{-1}, respectively). This result together with the blockade of I_{PB} by picrotoxin (Fig 1Ab) suggested that the I_{PB} in SDCN was carried by Cl^-.

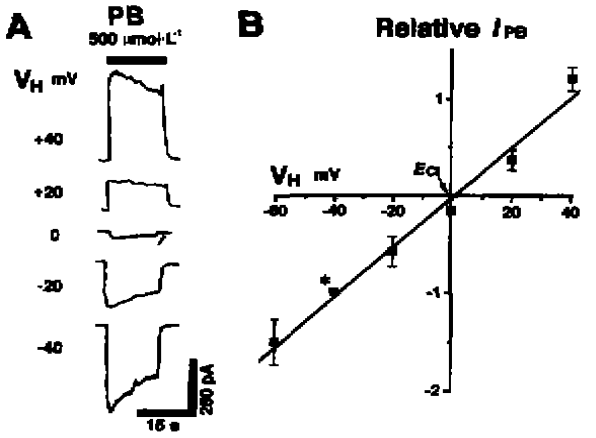

Fig 2. $I-V$ **relationship for PB-induced currents in SDCN. A: the currents induced by PB 500 μmol·L^{-1} at various holding potentials. B: the $I-V$ curve for PB-induced current. E_{Cl} represents theoretical Cl^- equilibrium potential. The asterisk (*) indicates the normalized control.**

Facilitatory effect of PB on GABA-induced Cl^- current PB augmented the GABA-induced inward current (I_{GABA}) in SDCN. Bicuculline, the competitive antagonist of $GABA_A$ receptors, suppressed the I_{GABA} in a concentration-dependent manner (n = 6), indicating that I_{GABA} in SDCN was mediated by activation of $GABA_A$ receptors. Since PB itself induced an inward Cl^- current (I_{PB}) at concentrations higher than 100 μmol·L^{-1} (Fig 1), the ratio of I_{GABA} enhancement by PB was calculated according to the following formula: ratio = $I_{(GABA+PB)}/(I_{GABA}+I_{PB})$. The I_{GABA} induced by GABA 3 μmol·L^{-1} was tested to avoid desensitization (Fig 3A). The I_{GABA} enhancement was detectable at PB 10 μmol·L^{-1} and reached the maximum at 300 μmol·L^{-1}. At higher concentrations, this effect was attenuated, leading to a bell-shaped concentration-response curve (Fig 3B).

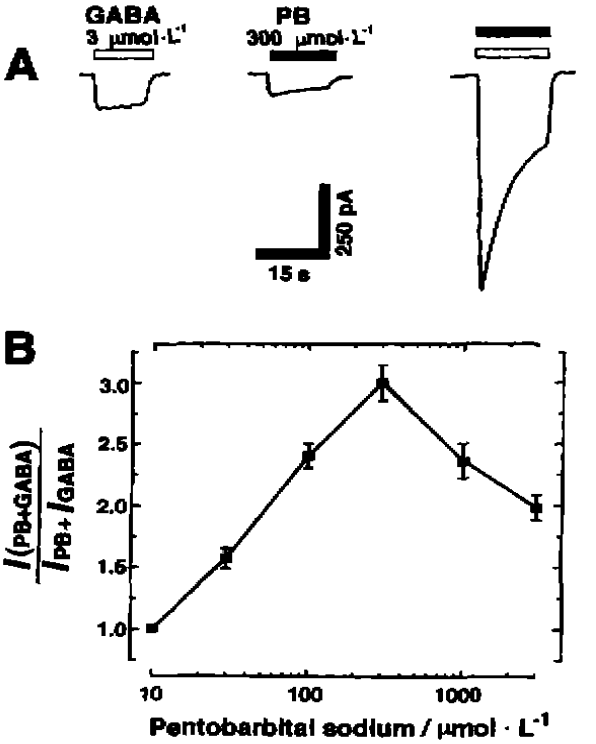

Fig 3. Facilitatory effect of PB on GABA-induced Cl^- current. A: the current responses induced by GABA, PB, and GABA + PB. B: the concentration-response curve of PB facilitation of Cl^- current induced by GABA 3 μmol·L^{-1}.

The concentration-response curves of I_{GABA} with or without PB 30 μmol·L^{-1} showed a parallel shift to the left without changes in I_{max} (Fig 4B). The EC_{50} (95 % confidence limits) values decreased from 6.9 (5.4 − 8.4) μmol·L^{-1} to 3.5 (2.9 − 4.1) μmol·L^{-1} (P < 0.01), whereas the Hill coefficient was not affected (1.05 in the presence of PB and 1.03 in the absence of PB). The result suggests that the

enhancement was mediated by an increase in apparent affinity of $GABA_A$ receptors for GABA. In addition, the magnitude of potentiation showed no voltage dependence either.

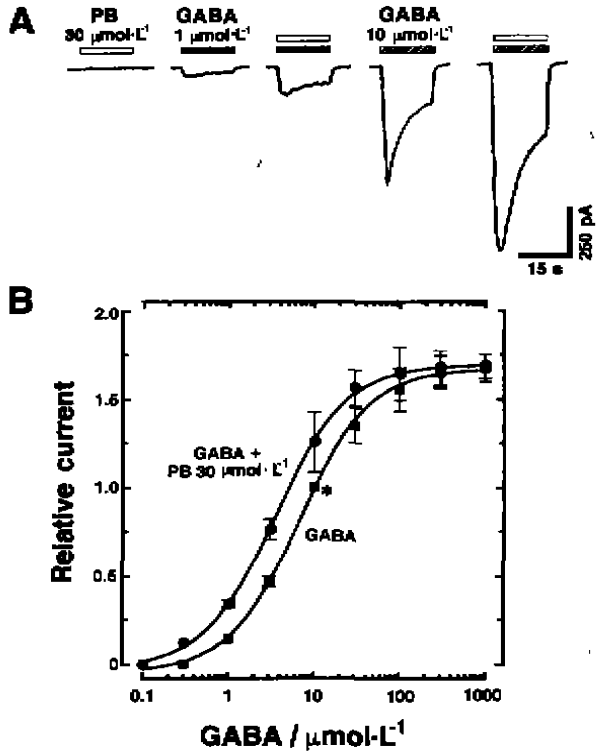

Fig 4. Effect of PB on the current responses to GABA in SDCN. A: the currents induced by PB, GABA and both PB and GABA. B: the concentration-response curves of GABA in the presence or in the absence of PB 30 μmol $\cdot L^{-1}$. The asterisk (*) indicates the normalized control.

DISCUSSION

PB is well known as one of the general anesthetics. PB can augment the GABA-induced currents and induce the Cl^- current in a variety of preparations[3-6]. However, at higher concentrations PB itself also suppressed the Cl^- channels[3]. In SDCN, PB induced an inward Cl^- current as well, and this effect was concentration-dependent. The *tail* (*hump*) current at wash out seems to be interpreted as the activation of undesensitized receptors by the agonist released from the blocked channels as discussed with pentobarbital-gated Cl^- currents in frog dorsal root ganglion cells[10].

A large body of evidence has shown a wide expression of $GABA_A$ receptor subtypes in the spinal cord including the SDCN region[11,12]. A recent study of murine β1, β2, and β3 subunits $GABA_A$ expressed in *Xenopus* oocytes demonstrated that PB and GABA might act at distinct ligand binding sites and/or utilize distinct transduction processes subsequent to ligand binding to open the Cl^- channel[13]. According to Cestari *et al*, the binding site of PB on $GABA_A$ receptor is on β subunits especially β2 and β3 subunits. In β3 homomeric receptors PB induced current in a dose-dependent manner. Thus, the immunohistochemical identification of the β2 and β3 subunits of the $GABA_A$ receptor in the mammalian spinal cord[12] together with the present data support the hypothesis that a unique binding site may be responsible for the agonistic effects of PB.

Using receptors expressed from mouse brain mRNA in *Xenopus* oocytes, Lin *et al*[14] found that enhancement of $GABA_A$ receptor-gated Cl^- channel response was a common action of structurally diverse anesthetics, suggesting that $GABA_A$ receptors play an important role in anesthesia. In the present study, the PB significantly potentiated $GABA_A$ receptor-mediated current. The mechanism of potentiation at the macroscopic current level was an increase in apparent affinity of $GABA_A$ receptorsfor GABA, without change in the efficacy or number of available receptor (I_{max}) or *voltage dependence* ($I - V$ *relationship*). It was quantitatively similar to that reported in frog dorsal root ganglion neurons. The underlying mechanism of enhancement was reported to be the increase in mean open time and burst duration of single $GABA_A$ receptor channels, with unaltered single channel conductance[15]. However, whether the same principle is applicable to SDCN remains to be studied.

In conclusion, we had demonstrated that PB had dual effects on SDCN: direct induction of a Cl^- *current through a unique binding site and* potentiation of $GABA_A$ receptor-Cl^- channel by increasing the apparent affinity of GABA for $GABA_A$ receptors. These results suggested that the anesthetic-induced changes at the level of the spinal cord could contribute significantly to the general anesthesia.

ACKNOWLEDGMENT To Misses KANG Jie-Fang and LI Hui-Li for their technical assistance.

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戊巴比妥对大鼠体外骶髓后联合核神经元的双重作用[1]

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关键词 戊巴比妥；GABA；膜片箝技术；神经元；脊髓

目的：研究戊巴比妥（PB）对急性分离的大鼠骶髓后联合核神经元（SDCN）的作用. **方法**：采用制霉菌素穿孔膜片箝技术. **结果**：（1）在箝制电位为 -40 mV 时，PB 以浓度依赖方式诱发内向电流. （2）印防己毒素可逆地阻断 PB 诱发的电流，其 EC_{50} 为 416（385 - 477）μmol·L^{-1}，Hill 系数为 1.08. （3）PB 诱发电流的逆转电位接近于 Cl^- 平衡电位. （4）PB 增大 GABA 诱发的 Cl^- 内流. 在 PB 30 μmol·L^{-1} 时，GABA 反应的 EC_{50} 由 6.9（5.4 - 8.4）μmol·L^{-1} 降至 3.5（2.9 - 4.1）μmol·L^{-1}. **结论**：PB 对 SDCN 的双重作用可能和全麻状态下脊髓水平的麻醉效应有关.

感　　谢

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