BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1998 May; 19 (3): 227 - 232

Isolation of rat sacral dorsal commissural neurons[1]

XU Tian-Le[2], PANG Zhi-Ping, KANG Jie-Fang, LI Ji-Shuo (*Department of Anatomy, K K Leung Brain Research Center, The Fourth Military Medical University, Xi-an 710032, China*)

KEY WORDS spinal cord; receptors; diazepam; neuroactive steroids; pentobarbital; substance P; 1-aminocyclopentane-1,3-dicarboxylate; patch-clamp techniques

AIM: To isolate rat sacral dorsal commissural neurons (SDCN). **METHODS**: Using enzymatic and mechanical dissociation techniques to isolate the neurons and using nystatin perforated patch technique to evaluate their functional state. **RESULTS**: The isolated neurons exhibited good responses to excitatory and inhibitory amino acids. The responses of SDCN to *N*-methyl-*D*-aspartate were markedly potentiated by substance P and *trans*-1-aminocyclopentane-1,3-dicarboxylate, whereas GABA responses were significantly potentiated by diazepam, pregnenolone, and pentobarbital. **CONCLUSION**: This preparation provides a satisfactory model for exploring the mechanisms of the SDCN in nociception and antinociception.

Mammalian sacral dorsal commissural nucleus, which represents the area just dorsal to the central canal in the lower lumbar and sacral spinal cord, is involved in nociception and antinociception. The sacral dorsal commissural neurons (SDCN) receive abundant afferent inputs from both the visceral and somatic organs. The convergence of the visceral and somatic inputs onto SDCN has been reported both electrophysiologically[1] and anatomically[2]. Nitric oxide synthase immunoreactivity in SDCN can be upregulated by peripheral visceral nerve injury[3]. By combining substance P receptor (SPR) immunohistochemistry with c-*fos*-like protein (FOS) immunohistochemistry technique, we recently found that SDCN expressed SPR. Furthermore, chemical irritation of the urinary bladder in the rat markedly increases the number of FOS immunoreactive neurons in the SDCN and the SDCN relay noxious information from the urinary bladder to the lateral parabrachial nucleus[4]. A number of putative neurotransmitter substances, including excitatory and inhibitory amino acids, peptides and monoamino acids, are present in SDCN[5]. The SDCN also integrate regulatory influences from the brain stem descending pathways. These characteristics thus make the SDCN a good tool to study the cellular mechanisms of the actions of numerous neuroactive substances[6,7]. In this study, we intended to find a fast and reliable method for isolating SDCN.

MATERIALS AND METHODS

Preparation The SDCN were acutely dissociated from 1 - 3-wk-old Sprague-Dawley rats, which were anesthetized with pentobarbitone-Na, and a laminectomy performed to expose the lower lumbar and sacral spinal cord together with dorsal roots. A segment about 10 - 15 mm long of lumbosacral (L_5 - S_2) spinal cord was dissected out and immersed into freezing standard external solution. After removal of the attached dorsal rootlets and the pia mater on the lateral aspects of the spinal cord, the spinal segment affixed with cyanoacrylic glue to a 15 mm × 15 mm agar block which just supported the spinal cord block. The nerve block together with the agar was positioned to the bottom of the cutting chamber of a vibratome tissue slicer (Dosaka, DTK-1000) with the dorsal surface of the spinal cord facing the vibrating blade. A cold standard external solution (about 5 ℃) bubbled with O_2 was consequently poured into the chamber to immerse the tissue block. The spinal segment was sectioned to yield several transverse slices 400 μm thick containing the SDCN region. The slices were preincubated in oxygenated standard external solution for 30 min at room temperature (22 - 25 ℃). Slices were treated enzymatically in oxygenated standard external solution containing pronase 0.18 $g \cdot L^{-1}$ at 32 ℃ for 20 min followed by exposure to thermolysin 0.18 $g \cdot L^{-1}$ for another 15 min in the

[1] Project supported by the National Natural Science Foundation of China, No 39770248.
[2] Phn 86-29-328-3229. Fax 86-29-324-6270.
E-mail patchclp@mail.fmmu.edu.cn
Received 1996-09-04 Accepted 1997-10-04

same conditions. After the enzyme treatment, the slices were kept in enzyme-free standard external solution for 1 h. A portion of SDCN region was micro-punched out with an electrolytically polished injection needle and transferred into a culture dish filled with standard external solution. Neurons were mechanically dissociated with fire-polished Pasteur pipettes under a phase-contrast microscope (Olympus, IX70) and a TV monitor (JVC).

Solutions The composition of normal standard external solution was NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, and glucose 10 mmol·L^{-1}. The pH was adjusted to 7.4 with tris-hydroxymethylaminomethane (Tris-base). The patch-pipette solution for nystatin perforated patch recording was KCl 150 and HEPES 10 mmol·L^{-1}. The pH was adjusted to 7.2 with Tris-base. The nystatin stock solution was prepared by dissolving nystatin in methanol at 10 g·L^{-1}. In general, nystatin 50 – 80 mg was dissolved in 5 – 8 mL methanol. After ultrasonication for 10 – 20 s, the pH value of the solution was lowered below 2.0 by HCl 1 mol·L^{-1} under constant stirring. This changed the turbid solution to a transparent one. Then the pH was elevated back to 7.2 by KOH 1 mol·L^{-1}. The stock solution was aliquotted in 1.0 mL centrifuge tubes and kept at −20 ℃. It kept its performing activity over 2 – 4 wk. The stock solution was diluted in the pipette solution (15 – 40 mL·L^{-1}) just before use to give a final nystatin concentration of 150 – 400 mg·L^{-1}. Although the solution was turbid due to supersaturating concentration (120 mg·L^{-1} seemed to be the saturating concentration), either the supernatant or the whole solution after vortex was used. The pipette solution with nystatin lost its activity within 3 – 5 h. The final concentration of methanol in the internal solution was 1.5 % – 4.0 %. Control experiments with methanol in the pipette solution in the absence of nystatin had no obvious effects on the neurons. All solutions were made up with a purified water (resistance > 18 MΩ·cm^{-1}) which was double-distilled and then membrane-filtered (Millipore).

Nystatin perforated whole-cell recording Electric measurement was carried out by using nystatin perforated patch recording configuration[3,4] under voltage-clamp condition at room temperature (22 – 25 ℃). Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm (Narishige) on a two-stage puller (Narishige, PB-7). The resistance between the recording electrodes filled with pipette solution and the reference electrode was 4 – 6 MΩ. The patch-pipette was positioned on a neuron using a hydraulic micromanipulator (Narishige, WR-3). The current and voltage were measured with a patch-clamp amplifier (Nihon Kohden, CEZ-2300), filtered at 1 kHz, and monitored on both a storage oscilloscope (Iwatsu Electronia, 5100A) and a pen recorder (San-ei, Omniace RT 3108). Axon DigiData 1200A and pCLAMP 6.0.2 program were used to produce signals, collect and process data. The membrane potential was held at −40 mV throughout the experiment. Measurement was started after the stabilization of the glycine response (15 – 25 min after cell attachment).

Drugs Quisqualic acid (QA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainic acid (KA), *N*-methyl-*D*-aspartic acid (NMDA), substance P (SP), *D*-2-amino-5-phosphonopentanoate (*D*-AP5), diazepam (Dia), pregnanolone (Pre), nystatin, and thermolysin were from Sigma; Pronase from Calbiochem, San Diego CA, USA; *trans*-1-aminocyclopentane-1, 3-dicarboxylate (tACPD) and 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) from Tocris Neuramin, Bristol, UK; *L*-glutamic acid (Glu) from Research Biochemicals International, Natick MA, USA. tACPD, CNQX, Dia, and Pre were dissolved in Me_2SO and then diluted in external solutions. Me_2SO did not induce any ionic current at the concentrations used. Other drugs were dissolved in external solutions. Drugs were applied by a pressure application system called the Y-tube[7], which allows a complete exchange of external solution surrounding a neuron within 10 – 20 ms.

Statistical analysis Experimental results were expressed as $\bar{x} \pm s$ and compared with *t*-test.

RESULTS

Morphology of isolated SDCN The commonest neurons found in SDCN were medium-sized (15 – 25 μm in diameter), fusiform cells with oval or triangular soma and 1 to 3 apical stem dendrites. Larger neurons (> 30 μm in diameter) were also found. The isolated SDCN

maintained 100 – 200 μm of the proximal dendrites, and the typical morphology revealed in the intact mammalian SDCN by intracellular horseradish peroxidase labeling[1,4]. The isolated neurons with long dendrites survived for 15 h after the isolation (Fig 1).

Excitatory amino acid-induced responses For recording NMDA-induced response, Glu and NMDA were applied to the SDCN immersed in Mg^{2+}-free external solution containing glycine 1 $\mu mol \cdot L^{-1}$. All tested neurons responded not only to Glu but also to QA, AMPA, KA, and NMDA. At a holding potential (V_H) of – 40 mV, Glu, QA, AMPA, KA, and NMDA evoked inward currents (Fig 2A). The currents increased in a sigmoidal fashion with increasing agonist concentration. The EC_{50} values (95 % confidence limits) were Glu 33 (25 – 41) $\mu mol \cdot L^{-1}$, NMDA 90 (77 – 103) $\mu mol \cdot L^{-1}$, QA 0.64 (0.41 – 0.87) $\mu mol \cdot L^{-1}$, AMPA 130 (109 – 151) $\mu mol \cdot L^{-1}$, and KA 96 (83 – 109) $\mu mol \cdot L^{-1}$. The Hill coefficients of the responses were 0.74, 0.83, 1.3, 1.1, and 1.3, respectively. The inward currents induced

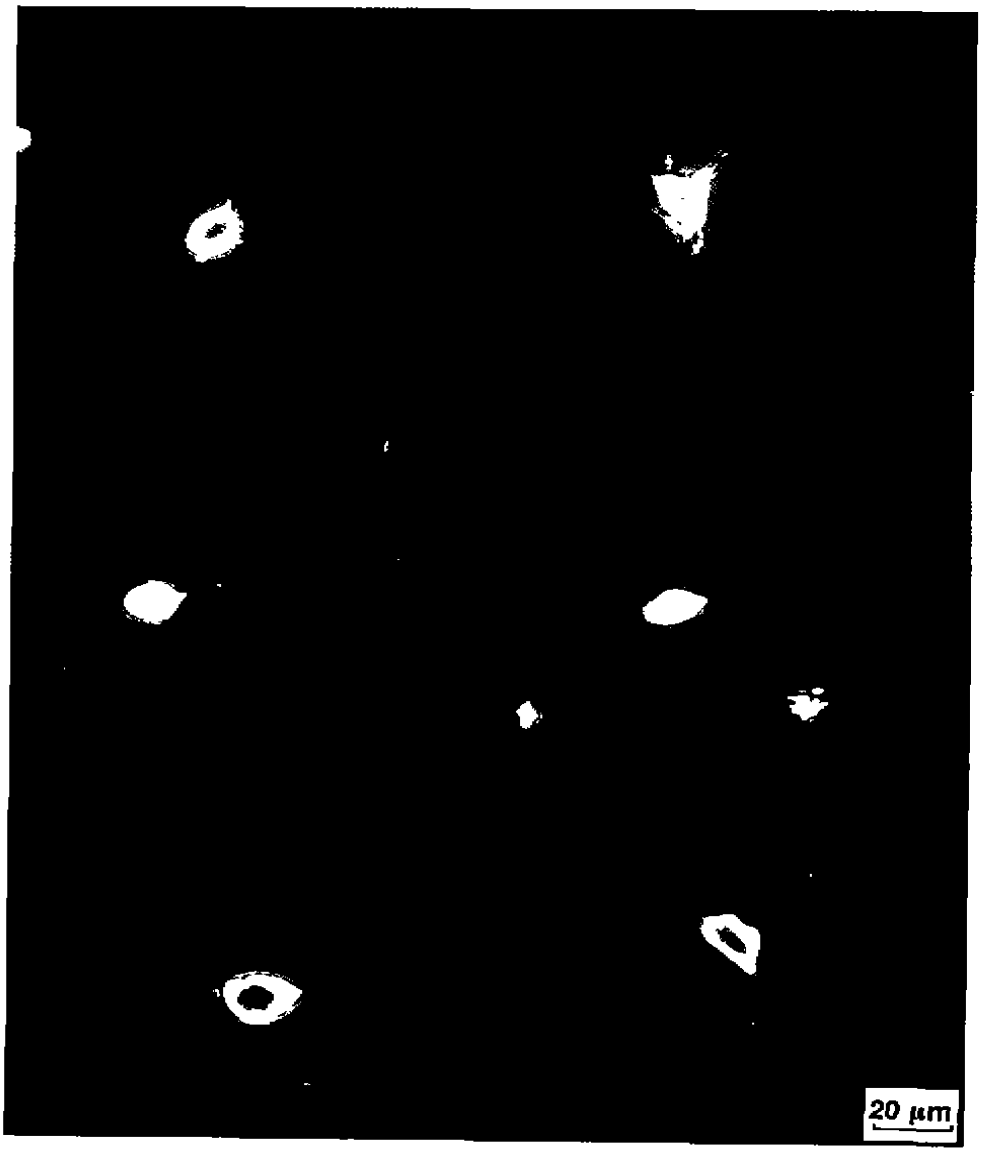

Fig 1. Isolated SDCN neurons from 14-d-old rats taken by phase-contrast optics.

by Glu, NMDA, QA, and AMPA exhibited an initial transient peak followed by a steadystate, while the KA-induced current usually exhibited only a steadystate (Fig 2A). In the presence of tACPD 10 μmol·L^{-1} (Fig 2Ba) and SP 10 μmol·L^{-1} (Fig 2Bb), the peak NMDA responses increased by 47 % ± 11 % ($P < 0.01$, $n = 5$) and 54 % ± 14 % ($P < 0.01$, $n = 5$), respectively. tACPD and SP *per se* evoked no response at the concentrations used.

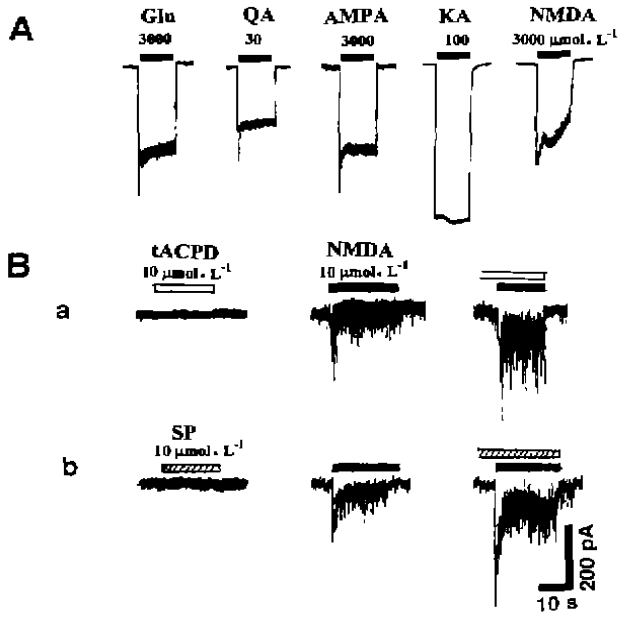

Fig 2. Excitatory amino acid-evoked responses in isolated SDCN neurons of 11- to 14-d-old rats. In zero-Mg^{2+} solution, the NMDA response was augmented by adding glycine 1 μmol·L^{-1}. V_H = − 40 mV. A: inward currents induced by Glu, QA, AMPA, KA, and NMDA. Horizontal bars indicate duration of amino acid applications. B: The NMDA (10 μmol·L^{-1}) response was potentiated by tACPD 10 μmol·L^{-1} (a) and SP 10 μmol·L^{-1} (b), respectively.

Inhibitory amino acid-induced responses

In the presence of high internal Cl^- (150 mmol·L^{-1}), GABA, glycine (Gly), and taurine (Tau) evoked inward currents. The GABA-, Gly-, and Tau-induced currents consisted of a transient initial peak and a successive steady-state component. The currents increased in a sigmoidal fashion with increasing agonist concentration. Fig 3 shows the inward currents induced by GABA. Its EC_{50} value (95 % confidence limits) and Hill coefficient were 5.2 (4.4 – 6.0) μmol·L^{-1} and 1.2, respectively. The GABA-induced response was potentiated by Dia, neuroactive steroids (pregnenolone, Pre) and pentobarbital (Pen) (Fig 3B). In the presence of Dia 0.1 μmol·L^{-1}, Pre 0.1 μmol·L^{-1}, and Pen 30 μmol·L^{-1}, the peak GABA responses increased by 71 % ± 16 % ($P < 0.01$, $n = 5$), 77 % ± 22 % ($P < 0.01$, $n = 5$), and 88 % ± 22 % ($P < 0.01$, $n = 5$), respectively.

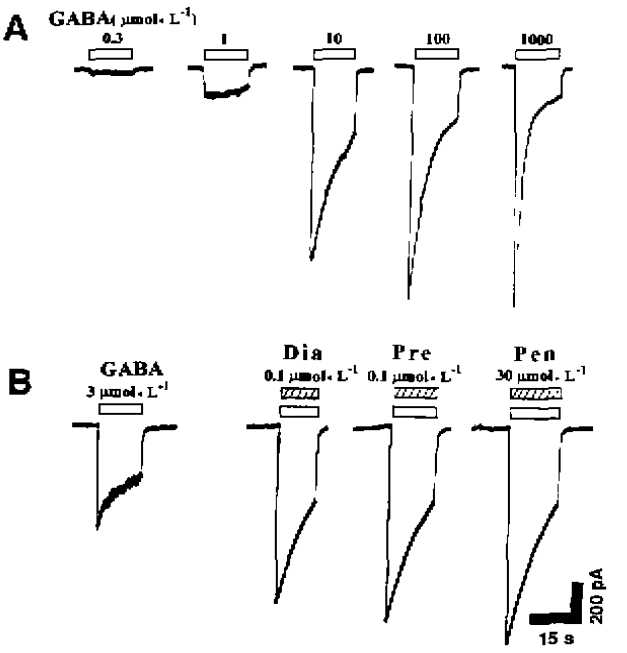

Fig 3. GABA-evoked responses. A: inward currents induced by GABA at various concentrations. Horizontal bars indicate duration of drug application. B: The GABA (3 μmol·L^{-1}) response was potentiated by Dia 0.1 μmol·L^{-1}, Pre 0.1 μmol·L^{-1}, and Pen 30 μmol·L^{-1}, respectively.

DISCUSSION

The responses to excitatory and inhibitory amino acids can be regularly observed in rat spinal dorsal horn neurons studied *in vivo*, in slice preparation and in cultured mouse spinal cord neurons[8,9]. However, in *in vivo* conditions and in slice preparation, it is difficult to analyze quantitatively the receptor activation because of the physical barrier to drug diffusion, and it is difficult to obtain reliable concentration-response data due to errors in estimating agonist concentrations following uptake of neurotransmitter agonists such Glu, or contributions from

endogenous high ambient levels of modulators such as Gly. The characteristics of receptors and ion channels expressed in cultured neurons are susceptible to environmental changes, so that cultured neurons may not reflect exactly the properties under *in vivo* conditions.

We have demonstrated here that isolated rat SDCN neurons pretreated with relatively low concentration of enzymes are responsively to excitatory amino acids (including NMDA) and inhibitory amino acids. The NMDA receptor-mediated current was enhanced by tACPD and SP, which is consistent to the previous studies in the spinal dorsal horn neurons[10,11]. Moreover, Dia, Pre, and Pen, which are potent allosteric modulators on the $GABA_A$ receptors[12], significantly potentiated the GABA response. These results indicate that the receptor-ionophore complexes for these agents are well-preserved in isolated SDCN neurons. Thus, the method provides a simple and cheap single cell preparation for exploring the function of the mammalian SDCN with natural conditions.

Several other reports using acutely isolated spinal cord neurons concentrated on the superficial dorsal horn[13]. Compared with the isolated superficial dorsal horn neurons, the neurons obtained in the present experiment are larger and survive longer, yet with numbers of cells to satisfy the needs of a typical patch clamp experiment. Thus, the isolated SDCN neurons are feasible to analyses of intracellular mechanisms[7,14]. Since a large number of putative endogenous neuroactive substances are present in SDCN region[5], this preparation could be useful for further studies of the actions of numerous substances present in the SDCN and the analysis of the intracellular second messenger-mediated processes, thus facilitating the exploration of the mechanisms of the SDCN in nociception and antinociception.

In the whole cell clamp experiments using isolated SDCN neurons with long dendrites, we cannot expect complete space clamp of these cells, especially for measurement of such voltage-gated fast currents as Na^+, K^+, and Ca^{2+} currents. However, the space clamp is much improved by decreasing the magnitude of the Na^+, K^+, or Ca^{2+} currents with decreasing extracellular Na^+, K^+, or Ca^{2+} concentrations. It should also be pointed out that these neurons are acceptable for the voltage-clamp studies of ligand-gated responses which are relatively small and very slow. Of course, we can easily isolate the intact SDCN neurons with only very short dendrites by rough pipetting. Thus, this preparation is also feasible to voltage-gated ionic currents with good space clamp[7].

REFERENCES

1 Honda CN. Visceral and somatic afferent convergence onto neurons near the central canal in the sacral spinal cord of the cat. J Neurophysiol 1985; 53: 1059 – 78.

2 Li JS, Qin BZ, Zheng HX, Du YJ, Ding YQ. The convergence of visceral and somatic primary afferent fibers onto the dorsal commissural nucleus of the sacral spinal cord. Abstracts of the Fourth IBRO World Congress of Neuroscience; 1995 Jul 9 – 14; Kyoto, Japan. New York: Rapid Communications Oxford; 1995. p 293.

3 Vizzard MA, Erdman SL, de Groat WC. Increased expression of neuronal nitric oxide synthase (NOS) in visceral neurons after nerve injury. J Neurosci 1995; 15: 4033 – 45.

4 Lu Y, Jin SX, Xu TL, Qin BZ, Li JS, Ding YQ, *et al*. Expression of *c-fos* protein in substance P receptor-immunoreactive neurons in response to noxious stimuli on the urinary bladder: an observation in the lumbosacral cord segments of the rat. Neurosci Lett 1995; 198: 139 – 42.

5 Sasek CA, Seybold VS, Elde RP. The immunohistochemical localization of nine peptides in the sacral parasympathetic nucleus and the dorsal gray commissure in the rat spinal cord. Neuroscience 1984; 12: 855 – 73.

6 Xu TL, Pang ZP, Li LS, Kang JF, Qin BZ. Excitatory amino acid-induced responses in rat sacral dorsal commissural neurons. Chin J Neuroanat 1996; 12: 261 – 72.

7 Xu TL, Nabekura J, Akaike N. Protein kinase C-mediated enhancement of glycine response in rat sacral dorsal commissural neurones by serotonin. J Physiol (Lond) 1996; 496: 491 – 501.

8 Yoshimura M, Jessell TM. Amino acid-mediated EPSPs at primary afferent synapses with substantia gelatinosa neurones in the rat spinal cord. J Physiol (Lond) 1990; 430: 315 – 35.

9 Yoshimura M, Nishi S. Primary afferent-evoked glycine- and GABA-mediated IPSPs in substantia gelatinosa neurones in the rat spinal cord *in vitro*. J Physiol (Lond) 1995; 482: 29 – 38.

10 Bleakman D, Rusin KI, Chard PS, Glaum SR, Miller RJ. Metabotropic glutamate receptors potentiate ionotropic glutamate responses in the rat dorsal horn. Mol Pharmacol 1992; 42: 192 – 6.

11 Urban L, Thompson SWN, Dray A. Modulation of spinal excitability: co-operation between neurokinin and excitatory amino acid neurotransmitters. Trends Neurosci 1994; 17: 432 – 8.

12 Shimura M, Harata N, Tamai M, Akaike N. Allosteric modulation of $GABA_A$ receptors in acutely dissociated neurons of the suprachiasmatic nucleus. Am J Physiol, 1996; 270 (Cell Physiol 39): C1726 – 34.

13 Arancio O, Murase K, Yoshimura M, MacDermott AB. Heterogeneous distribution of excitatory amino acid receptors on postnatal neurons acutely dissociated from rat dorsal horn. Neuroscience 1993; 52: 159 – 67.

14 Xu TL, Akaike N. Suppression of NMDA response in rat sacral dorsal commissural neurons by AMPA receptor activation: a study by the nystatin perforated patch technique. Chin J Neuroanat 1996; 12: 349 – 60.

227-232

大鼠骶髓后连合核神经元的分离[1] R441.1

徐天乐[2]，庞志平，康杰芳，李继硕 （第四军医大学

解剖学教研室，梁铼琚脑研究中心，西安 710032，中国）

关键词 脊髓；受体；地西泮；神经活性留类；戊巴比妥；P物质；1-氨基环戊基-1,3-二羧酸；膜片箝技术

目的：急性分离大鼠骶髓后连合核神经元。**方法**：采用酶消化结合机械性分离技术分离神经元，以制霉菌素穿孔膜片箝技术检测其机能状态。**结果**：分离的神经元对兴奋性和抑制性氨基酸具有良好的反应。P物质(SP)和反式-氨基环戊基-1,3-二羧酸(tACPD)明显增强其 NMDA 反应。而地西泮、孕烯诺龙和戊巴比妥存在下，GABA 反应被显著加强。**结论**：急性分离的大鼠 SDCN 神经元为探索 SDCN 参与痛和镇痛的机制提供了理想模型。

BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1998 May; 19 (3): 232 - 237

Analysis of multidrug effects by parameter method[1]

ZHENG Qing-Shan[2], SUN Rui-Yuan[3]
(*Institute of Clinical Pharmacology, Yijishan Hospital, Wannan Medical College, Wuhu 241001*; [3]*Institute of Materia Medica, Wannan Medical College, Wuhu 241001, China*)

KEY WORDS drug synergism; drug antagonism; combination drug therapy; biometry

AIM: To set up a new analytic method for multidrug effects. **METHODS**: Based on the principles of the target site kinetics and the equieffective test, a new mathematical model was set as $Q = (E_o - E_e)/|E_e \cdot W - s_{\bar{x}} \cdot T|$ ($-1 < Q < 1$ addition, $Q \leqslant -1$ antagonism, $Q \geqslant 1$ synergism) where E_o = a fitted value of the observed effect of a combination, E_e = an expected value of combined effect, W = an equieffective criterion decided by a special field, $s_{\bar{x}}$ = a common standard error of E_o and E_e, and T = a value of one-sided $t_{0.05}$. All the calculations were completed with computer. Dose-effect data from different types of experiments were fitted by the new model and the results were compared with those of other methods. **RESULTS**: This parameter method dealt with different types of data well fitted with the Hill equation, and was not limited to analyze receptor interaction of drugs, or the number of combined drugs. A series of Q values was obtained from all levels of dose-effect for a systematic analysis. The analysis took the criterion of a special field and laboratory error into account. **CONCLUSION**: This parameter method can effectively analyze the multidrug effects.

Quantitative analysis of the combined effects of multidrugs, such as synergism, antagonism, and addition, is not fully solved, especially quantitative data with more than 2 drugs in combination, or in large animal experiment and clinical trials. To qualitative data, Chou-Talalay combination index method[1, 2], Xu's method[3] and Jin's method[4] are widely used on basis of different background (mechanism or empirical), but those methods may yield different or even opposite results. Now, it becomes imperative to solve the problem to decide new multidrug clinical therapeutic design.

It is generally accepted that dose-effect curve of sigmoid or hyperbola shape can be expressed with Hill equation and is characterized by its pharmacodynamic parameters. The parameters in different combinations must vary with the results of the combined effect. In this paper, we try to find a new analytic method from the regularity of the parameters changes.

ANALYSIS OF THE PARAMETER METHOD

Formula

$$Q = \frac{E_o - E_e}{|E_e \cdot W - s_{\bar{x}} \cdot T|} \quad (1)$$

[1] Project supported by the National Natural Science Foundation of China, No 39670845 and the Science Foundation of Anhui Province Educational Commission, No 96JL0117.
[2] Correspondence to ZHENG Qing-Shan, MS.
Phn 86-553-587-7855. Fax 86-553-586-3079.
Received 1997 - 05 - 16 Accepted 1998-03-17