研究報告書

厚生科学研究費補助金（長寿科学総合研究事業）

分担研究報告書-2

G.研究発表

1.論文発表

○Hiruma H, Saito A, Ichikawa T, Kiriyama Y, Hoka S, Kusakabe T, Kobayashi H, Kawakami: Effects of substance P and calcitonin gene-related peptide on axonal transport in isolated and cultured adult mouse dorsal root ganglion neurons. Brain Res 883:184-191,2000

○Hiruma H, Ichikawa T, Kobayashi H, Hoka S, Takenaka T, Kawakami T.: Prostaglandin E(2) enhances axonal transport and neuritogenesis in cultured mouse dorsal root ganglion neurons. Neuroscience 100:885-891 2000

○ Kusakabe T, Matsuda H, Hirakawa H, Hayashida Y, Ichikawa T, Kawakami T, Takenaka T.: Calbindin D-28k immunoreactive nerve fibers in the carotid body of normoxic and chronically hypoxic rats. Histol Histopathol. 15:1019-1025, 2000

○Kusakabe T, Yoshida T, Matsuda H, Yamamoto Y, Hayashida Y, Kawakami T, Takenaka T.: Changes in the immunoreactivity of substance P and calcitonin gene-related peptide in the laryngeal taste buds of chronically hypoxic rats.Histol Histopathol. 15:683-688, 2000

○Ichikawa T, Ishihara K, Kusakabe T, Hiruma H, Kawakami T, Hotta K. : CGRP modulates mucin synthesis in surface mucus cells of rat gastric oxyntic mucosa. Am J Physiol Gastrointest Liver Physiol. 279: G82-89, 2000

○ Kusakabe T, Hayashida Y, Matsuda H, Kawakami T, Takenaka T.: Changes in the peptidergic innervation of the rat carotid body a month after the termination of chronic hypoxia. Adv Exp Med Biol. 475: 793-799, 2000

○Matsuda H, Kusakabe T, Hayashida Y, Powell FL, Ellisman MH, Kawakami T, Takenaka T.: Morphological adaptation of the peptidergic innervation to chronic hypoxia in the rat carotid body.Adv Exp Med Biol. 475: 623-630, 2000

2.学会発表

○Yamamoto M, Hiruma H, Hosokawa R, Nishiba S, Takenaka T, Kawakami T: The newrotrophins brain-derived neurotrophic factor and hepato cyte growth factor increase axonal transport in untreated and glutamate-treated cultured rat motor neurons. Society for Neuroscience 30th Annual Meeting 2000 L.A., USA

○Hiruma H Nagashima R, Sakai R, Shintani M, Kono T, Kawakami T.: Glutamate inhibits axonal transport in cultured rat hippocampal neurons via NMDA and AMPA receptors. Society for Neuroscience 30th annual meeting 2000 L.A., USA

○ Kiriyama Y, Hiruma H, Yamamoto M, Kobayashi TTomita T, Kawakami T: NMDA induced inhibition of hippocampal axonal transport is mediated by nitric oxide. Society for Neuroscience 30th annual meeting 2000 L.A., USA

○島田純一,比留間弘美,喜里山順子,西田早苗,竹中敏文,川上　倫;NMDA 受容体を会する海馬ﾆｭｰﾛﾝ軸索輸送抑制における一酸化窒素の役割　第 78 回日本生理学大会 2001 京都

○山本美絵,比留間弘美,西田早苗,片倉　隆,竹中敏文,川上　倫;BDN 下による運動ﾆｭｰﾛﾝ軸索輸送促進作用を仲介する受容体と細胞内ｼｸﾞﾅﾙ伝達　第 78 回日本生理学大会 2001 京都

○日下部辰三,林田嘉朗,川上　倫,竹中敏文;低酸素環境における頚動脈小体のﾍﾟﾌﾟﾁﾄﾞ性神経支配に対する二酸化炭素分圧の効果」第 78 回日本生理学大会 2001 京都

○比留間弘美,西田早苗,日下部辰三,竹中敏文,川上倫;培養海馬ﾆｭｰﾛﾝ軸索輸送に及ぼすｸﾞﾙﾀﾐﾝ酸の影響　第 78 回日本生理学大会 2001 京都

H.知的所有権の取得状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

（資料4）　ラット後根神経節細胞の軸索輸送に対する
リドカインの効果

(Anesthesiology in press 2001 manuscript)

Low Concentration Lidocaine Rapidly Inhibits Axonal Transport in Cultured Mouse Dorsal Root Ganglion Neurons

Akifumi Kanai M.D., * Hiromi Hiruma M.D., † Takashi Katakura, ‡ Sumi Sase M.D., * Tadashi Kawakami M.D., § Sumio Hoka M.D. ||
Departments of Anesthesiology and Physiology, Kitasato University School of Medicine, Sagamihara, Japan.

* Resident, Department of Anesthesiology, Kitasato University School of Medicine, Sagamihara, Japan.
† Associate Professor, Department of Physiology, Kitasato University School of Medicine, Sagamihara, Japan.
‡ Assistant, Department of Physiology, Kitasato University School of Medicine, Sagamihara, Japan.
§ Professor, Department of Physiology, Kitasato University School of Medicine, Sagamihara, Japan.
|| Professor, Department of Anesthesiology, Kitasato University School of Medicine, Sagamihara, Japan.

Correspondence: Dr. Hiromi Hiruma, Department of Physiology, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara 228-8555 Japan, TEL: +81-42-778-9159, FAX: +81-42-778-9841, E-MAIL: hiruma@med.kitasato-u.ac.jp
Supported by the Academic Frontier Project of the Ministry of Education, Science, Sports and Culture, Japan, and by Comprehensive Research on Aging and Health (H10-Aging and Health-008), Health Science Research Grants, Ministry of Health and Welfare, Japan.
Presented in part at the 77th meeting of the Physiological Society of Japan, Yokohama, Japan, March 27, 2000, at the 47th annual meeting of the Japan Society of Anesthesiologists, Tokyo, Japan, April 7, 2000, and at the 2000 annual meeting of the American Society of Anesthesiologist, San Francisco, California, October 17, 2000.

Abstract

Background: Axonal transport plays a critical role in supplying materials for a variety of neuronal functions such as morphogenetic plasticity, synaptic transmission, and cell survival. In the present study, we investigated the effects of the analgesic agent lidocaine on axonal transport in neurites of cultured mouse dorsal root ganglion (DRG) neurons. In relation to their effects, the effects of lidocaine on the growth rate of the neurite were also examined.
Methods: Isolated mouse DRG cells were cultured for 48 h until full growth of neurites. Video-enhanced microscopy was used to observe particles transported within neurites and to measure the neurite growth under control condition and in the presence of lidocaine.
Results: Application of 30 (M lidocaine immediately reduced the number of particles transported in anterograde and retrograde axonal directions. These effects were persistently seen during the application (26 min) and were reversed by lidocaine wash-out. The inhibitory effect was dose-dependent at concentrations from 0.1 to 1000 (M (IC50: 10 (M). In Ca2+-free extracellular medium, lidocaine failed to inhibit axonal transport. Calcium ionophore A23187 (0.1 (M) reduced axonal transport in both directions. The inhibitory effects of lidocaine and A23187 were abrogated by 10 (M KN-62, a Ca2+/calmodulin-dependent protein kinase II (CAM II kinase) inhibitor. Application of such low concentration lidocaine (30 (M) for 30 min reduced the growth rate of neurites, and this effect was also blocked by KN-62.
Conclusions: Low concentration lidocaine rapidly inhibits axonal transport and neurite growth via activation of CAM II kinase.

Introduction

Axonal transport plays a critical role in supplying materials for a variety of neuronal functions such as morphogenetic plasticity,1-3 synaptic transmission,3-6 and cell survival.7,8 Lidocaine, as an analgesic agent, is known to have direct neuronal actions such as inhibition of Na+ action potential generation9-11 and conduction,12 and depression of synaptic transmission.13,14 There have been also some reports with regard to the lidocaine effect on axonal transport. While axonal transport of catecholamine-synthesizing enzymes in vivo has been reported to be unaffected by lidocaine even at high concentrations (0.5-1% = 18.5-37 mM),15 this local anesthetic has shown to inhibit fast axonal transport of labeled proteins both in vivo and in vitro preparations.16-20 The latter also used high concentrations (0.1-0.6% = 4-22 mM),17-20 and thus such an inhibitory effect on axonal transport has been recognized as neurotoxic side effect of nerve blocking.18 In the present study, we focused on assessing the effect of low concentration lidocaine on axonal transport in cultured sensory neurons by using video-enhanced microscopy that allow us to observe the real-time dynamics of axonal transport. In relation to their effects, the effects of lidocaine on the growth rate of the neurite were also examined.

Materials and Methods

Cell Culture

Animal use in this study was approved by the Animal Experimentation and Ethics Committee of Kitasato University School of Medicine, Sagamihara, Japan. Adult male c57BL/6 mice (8-week old) were sacrificed with ether and the dorsal root ganglia were removed. The ganglia were immersed immediately in Hams' F-12 culture medium (GOBCO BRL, Grand Island, NY), and incubated for 90 min at 37° C in Hams' F-12 medium containing 2 mg/ml collagenase (Worthington Biochemical, Freehold, NJ). Subsequently, the ganglia were incubated for 15 min at 37° C in Ca2+- and Mg2+-free Hanks' balanced salt solution (g/l: KCl, 0.4; KH2PO4, 0.06; NaCl, 8; Na2PO4/7H2O, 0.09; glucose, 1; phenol red, 0.01; HEPES, 3.6; and NaOH, 0.3) containing 2.5 mg/ml trypsin (Sigma Chemical Co., St. Louis, MO). Trypsin activity was then inhibited by the addition of 0.125 mg/ml trypsin inhibitor (Sigma). After a rinse with enzyme-free Hams' F-12 medium, the ganglia were triturated using fire-polished pipettes (inner diameter: 0.2-0.5 mm). The isolated cells were plated onto polylysine-coated coverglasses and cultured for 48 h at 37° C in Hams' F-12 medium containing 10% fetal bovine serum and penicillin (100 units/ml)-streptomycin (100 (g/ml) under 5% CO2 (pH 7.4).

Experimental Cell Preparation

The coverglass on which cells were cultured was attached with waterproof tape to the underside of a 0.5-mm-thick stainless plate (50 x 80 mm) with a lozenge-shaped hole (25 x 35 mm). The topside of the steel plate was covered with another coverglass, leaving small opening on both sides to inject solutions. The culture medium was then replaced with physiological salt solution (PSS; see below) (37° C). The plate was mounted onto the stage of an inverted Zeiss Axiomat microscope (Carl Zeiss, Oberkochen, Germany), with an oil-immersed planapochromat 64x objective (Carl Zeiss). The stage was maintained at 37° C. The drug-containing solution was injected into one opening using a Pasteur pipette, and the solution spilling from the other opening was removed by a suction pump.

Solutions and Drugs

The composition of PSS (pH 7.4) was 135 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 5.5 mM glucose (all from Wako Pure Chemical, Osaka, Japan). Calcium (Ca2+)-free solution was prepared by excluding Ca2+ from PSS and adding 2 mM EGTA (Wako Pure Chemical). Lidocaine hydrochloride (Research Biochemical International, Natick, MA) was directly dissolved in PSS (pH 7.4). Calcium ionophore A23187 (Sigma) and KN-62 (Biomol, Plymouth Meeting, PA) were each dissolved in dimethyl sulfoxide (DMSO, Sigma) and then diluted with aqueous solution. The DMSO concentration was 0.01% and DMSO at this concentration had no effect on axonal transport and neurite growth.

Observation of Axonal Transport

Nomarski images of neurites (length > 100 (m, width > 1 (m) obtained by inverted microscopy were transformed into video images with enhanced contrast by using a video camera (Harpicon, Hamamatsu Photonics, Hamamatsu, Japan) and a video image enhancement system (DVS-20, Hamamatsu Photonics). Serial video images were displayed on a video monitor (C1864, Hamamatsu Photonics) and stored on a video recorder (PVW-2800, Sony, Tokyo). This processing allowed observation of living cells magnified approximately 10,000 times on the video monitor. Axonal transport was estimated on the video monitor by counting the number of particles (diameter > 50 nm) crossing the line drawn perpendicular to the long axis of the neurites. Counts were made for 2 min at 3-min intervals during periods before and after the injection of drugs.

Measurements of Neurite Growth Rate

Dorsal root ganglion (DRG) cells cultured for 48 h were prepared for measurements of neurite growth rate as described above. The chamber was filled with PSS and maintained at 37° C. Length of neurites (width > 1.0 (m) was measured just before and 30 min after treatment with drugs under video-enhanced microscopy at ×3,000 magnification. In control cells, PSS was applied.

Statistical analysis

Data from experiments on axonal transport are expressed as mean + SD and reported as percentage of the control value (before drug application). Analysis of variance (ANOVA) was used to evaluate the statistical significance of fluctuations over time. Differences between the control and values obtained during application of test agents were examined for statistical significance by Student's paired t-test. Neurite growth rates are expressed as mean + SD. The statistical significance of difference in the growth rate between control (non-treated cells) and treated cells was determined by Student's t-test.

Results

Video-enhanced microscopy displayed the movement of particles toward the axon terminal (anterograde) and back to the cell body (retrograde) (video 1). Some of the transported particles appeared to be mitochondria and lysosomes according to their microscopic morphology. In the control extracellular medium (PSS, pH 7.4, 37° C), the mean numbers of particles (per minute) transported in anterograde and retrograde directions were 68.3 + 17.9 (mean + SD, n = 40) and 68.7 + 18.2 (n = 40), respectively. Length of the neurites used for the experiments on axonal transport was ranging from 100 to 360 μm.

Effects of Low Concentration Lidocaine on Axonal Transport

Application of lidocaine at a low concentration (30 (M) for 10 min resulted in an immediate but reversible decrease in the number of particles transported in both the anterograde and retrograde directions (fig. 1). Application of lidocaine at the same concentration (30 (M) but for a longer period (26 min) resulted in a significant decrease in the number of transported particles during the application (video 1A, fig. 2A). Maximum inhibition of particle transfer amounted to 60% of the control at 8 min after the start of application, reaching a plateau for the remaining period of the experiment (fig. 2A). Application of lidocaine at concentrations ranging between 0.1-1000 μM indicated that the effect of the drug on axonal transport was dose-dependent (fig. 3). The median inhibitory concentration (IC50) was 10 (M for both the anterograde and retrograde axonal transport.

Effects of Low Concentration Lidocaine on Axonal Transport in Ca2+-Free Extracellular Medium

In Ca2+-free extracellular medium (with 2 mM EGTA), 30 (M lidocaine failed to decrease the number of particles in either anterograde or retrograde direction (video 1B, fig. 2B). These results suggest that extracellular Ca2+ is required for the inhibition of axonal transport induced by lidocaine.

Effects of Activation of CAM II Kinase on Axonal Transport

Failure of lidocaine to inhibit axonal transport in the absence of extracellular Ca2+ suggests that accumulation of Ca2+ inside the cell might be involved in lidocaine-induced inhibition of axonal transport. Ca2+/calmodulin-dependent protein kinase II (CaM II kinase), which is activated by intracellular Ca2+, has been implicated in regulating the organization of neuronal cytoskeleton21 and neurite growth.22-24 We therefore hypothesized that CaM II kinase activity is involved in the lidocaine-induced inhibition of axonal transport. First, we investigated the effect of activation of CaM II kinase by Ca2+ ionophore on axonal transport. The Ca2+ ionophore A23187 (0.1 (M) decreased the number of particles transported in anterograde and retrograde directions (video 1C, fig. 4A). These inhibitory effects were blocked by the presence of CaM II kinase inhibitor KN-62 (10 (M) in the extracellular medium (video 1D, fig. 4B), whereas KN-62 alone did not have any effect on axonal transport (data not shown, n = 4) as described in our previous study.25 These results indicate that the activation of CaM II kinase leads to inhibition of axonal transport in cultured DRG neurons.

Effects of Low Concentration Lidocaine in the Presence of CAM II Kinase Inhibitor

In the presence of the CaM II kinase inhibitor KN-62 (10 (M) in extracellular medium, 30 (M lidocaine failed to suppress axonal transport in either anterograde or retrograde direction (video 1E, fig. 2C). Thus, the inhibitory effect of low concentration lidocaine seems to be mediated by activation of CAM II kinase.

Effects of Low Concentration Lidocaine on Neurite Growth

Axonal transport is known to relate to morphogenetic plasticity.1-3 Therefore, we further attempted to examine the effects of low concentration lidocaine on neurite growth. As indicated in table 1, treatment of DRG neurons with 30 (M lidocaine for 30 min resulted in a significant reduction in the growth rate of neurites, relative to the control. This effect of lidocaine was also abrogated by the addition of 10 (M KN-62, while KN-62 alone did not affect neurite growth (table 1).

Discussion

Using video-enhanced microscopy, we show that lidocaine at low concentrations (0.1-1000 μM: IC50 = 10 μM) resulted in a rapid decrease in the number of particles in both anterograde and retrograde directions. Previous studies in which labeled proteins were measured showed that lidocaine inhibited axonal transport when used at high concentrations of 0.1-0.6% (3.7-22 mM) but not at lower concentrations.17-20 Such high concentrations of lidocaine are also known to cause destruction of microtubules, thus resulting in an irreversible arrest of axonal transport.18,19 The present study shows for the first time that lidocaine, even at low concentrations, significantly and reversibly inhibits axonal transport.

We next investigated the mechanisms mediating the inhibitory action of low concentration lidocaine. Here we demonstrated that inhibition of axonal transport induced by lidocaine was completely blocked when we used Ca2+-free extracellular medium. Therefore, extracellular Ca2+ may be a requisite for inhibition of axonal transport. In addition, we demonstrated here that Ca2+ ionophore A23187 inhibited axonal transport. These results suggest that the lidocaine-induced inhibition of axonal transport may result from the accumulation of Ca2+ inside the cell. This hypothesis needs to be supported by further studies on intracellular signaling mechanisms triggered by an increase in [Ca2+]i. Previous biochemical studies have shown that CAM II kinase phosphorylates microtubule-associated proteins (MAPs), MAP2 and tau protein, leading to microtubule disassembly,21 and that the latter causes inhibition of fast axonal transport.1 In turn, CAM II kinase is activated by intracellular Ca2+. Therefore, we postulated that activation of CAM II kinase might result in inhibition of axonal transport. In fact, we found that the inhibitory effect of Ca2+ ionophore A23187 was abrogated by CAM II kinase inhibitor KN-62. The effect of low concentration lidocaine was also blocked by KN-62. Thus, the lidocaine-induced inhibition of axonal transport appears to be mediated by activation of CAM II kinase. However, our findings are likely to be inconsistent with previous [Ca2+]i measurement and electrophysiological studies on DRG cells. The Ca2+ indicator fura-2-determined [Ca2+]i in rat DRG cells has shown to increase in response to lidocaine, but this increase can be detected at lidocaine concentrations > 3 mM (EC50: 21 mM),26 which are much higher than those used in the present study. Moreover, lidocaine has been demonstrated to inhibit Ca2+ current in frog DRG cells at a threshold

concentration of 10 μM.27 Therefore, lidocaine at low concentration is likely to reduce the influx of Ca2+ through Ca2+ channel mechanism. One possibility to explain the inconsistency between their findings and ours is that lidocaine might affect Ca2+ pump or passive Ca2+ influx through cell membrane to modulate intracellular Ca2+ signaling. Garcia-Martin and Gutierrez-Merino28,29 and Garcia-Martin et al. 30 have demonstrated that lidocaine acutely inhibits Ca2+ pump in synaptosomal plasma membrane, and thus suggested that lidocaine can increase the Ca2+ level of neuronal cytosol. They also mentioned that the lidocaine concentration needed to produce ~50% inhibition of Ca2+ pump activity (K0.5) is 0.44 mM, but that, due to the dependence of local anesthetic-lipid membrane interaction on membrane potential, this K0.5 value should be lower at the cell resting membrane potential.30 Therefore, it might be possible that lidocaine at low concentrations induces the accumulation of intracellular Ca2+ by inhibiting Ca2+ pump activity under normal extracellular Ca2+ concentration condition. However, further studies are required to address this issue.

We further discuss here the relationship between Ca2+ and axonal transport. A number of studies have shown that fast axonal transport in a variety of neuronal types is reduced under the Ca2+-free extracellular condition.31-37 The intraneuronal injection of Ca2+ chelater has also shown to exhibit the same effect.38 Curiously, the intraneuronal injection of Ca2+ blocks axonal transport, also.38 Furthermore, it has been reported that axonal transport is inhibited by Ca2+ ionophores39-41 and by chemical agents that raise concentrations of intracellular Ca2+,42 which is similar to our results. Taken together, not only reduction but also elevation in intracellular Ca2+ levels appears to be a factor to inhibit axonal transport. Thus, the elevation of intracellular Ca2+ levels could be the acceptable mechanism in mediating inhibitory action of lidocaine on axonal transport.

In order to know the relevance of lidocaine-induced axonal transport to neurite growth, we also investigated the effects of low concentration lidocaine on neurite growth. We found that treatment of lidocaine at a low concentration (30 (M) for 30 min inhibited the growth rate of neurites. These results are similar to our previous findings that lidocaine at low concentrations reduces sprouting DRG cells in the process of culture.43 Here, we further show that lidocaine is also effective to inhibit the growth of neurites already present, implying that the inhibition of neurite growth is related to prevention of axonal transport. Furthermore, we demonstrated that the inhibitory effect of lidocaine on neurite growth was blocked by KN-62 (10 (M). This is supported by previous studies showing that the overexpression of CAM II kinase inhibits neurite growth.22-24 Our present findings suggest that the lidocaine-induced inhibition of neurite growth is mediated by activation of CAM II kinase, which is similar to its inhibition of axonal transport. Thus, lidocaine may simultaneously inhibit axonal transport and neurite growth by activating CAM II kinase in sensory neurons.

In summary, we demonstrated in the present study that lidocaine at low concentrations inhibited axonal transport and neurite growth. These inhibitory actions are mediated through activation of CAM II kinase.

References

1. Grafstein B, Forman DS: Intracellular transport in neurons. Physiol Rev 1980; 60:1167-283

2. Grafstein B, Burmeister DW, McGuinness CM, Perry GW, Sparrow JR: Role of fast axonal transport in regeneration of goldfish optic axons. Prog Brain Res 1987; 71:113-20

3. Cull RE: Role of axonal transport in maintaining central synaptic connections. Exp Brain Res 1975; 24:97-101

4. Fernandez HL, Donoso JA: Axonal transport involvement in long-lasting synaptic modifications in Blatta orientalis. J Neurobiol 1983; 14:61-75

5. Holmgren E, Karlsson JO, Sjostrand J: Changes in synaptic function induced by blockage of axonal transport in the rabbit optic pathway. Brain Res 1978; 157:267-76

6. Gorio A: The role of axoplasmic transport in the restoration of synaptic transmission and in the process of sprouting during nerve regeneration. Adv Exp Med Biol 1987; 209:41-9

7. Blaser PF, Clarke PG: Timing of neuronal death following successive blockade of protein synthesis and axoplasmic transport in the axonal target territory. Dev Neurosci 1992; 14:271-7

8. Catsicas M, Pequignot Y, Clarke PG: Rapid onset of neuronal death induced by blockade of either axoplasmic transport or action potentials in afferent fibers during brain development. J Neurosci 1992; 12:4642-50

9. Wagman IH, De Jong RH, Prince DA: Effects of lidocaine on the central nervous system. ANESTHESIOLOGY 1967; 28:155-72

10. Narahashi T, Yamada M, Frazier DT: Cationic forms of local anaesthetics block action potentials from inside the nerve membrane. Nature 1969; 223:748-9

11. Omana-Zapata I, Khabbaz MA, Hunter JC, Bley KR: QX-314 inhibits ectopic nerve activity associated with neuropathic pain. Brain Res 1997; 771:228-37

12. Fink BR, Cairns AM: Differential slowing and block of conduction by lidocaine in individual afferent myelinated and unmyelinated axons. ANESTHESIOLOGY 1984; 60:111-20

13. Sotgiu ML, Lacerenza M, Marchettini P: Selective inhibition by systemic lidocaine of noxious evoked activity in rat dorsal horn neurons. Neuroreport 1991; 2:425-8

14. Kuno M, Matsuura S: Sites and mechanisms of action of lidocaine upon the isolated spinal cord of the frog. Brain Res 1982; 249:87-93

15. Ngai SH, Dairman W, Marchelle M: Effects of lidocaine and etidocaine on the axoplasmic transport of catecholamine-synthesizing enzymes. ANESTHESIOLOGY 1974; 41:542-8

16. Fink BR, Kish SJ: Reversible inhibition of rapid axonal transport in vivo by lidocaine hydrochloride. ANESTESIOLOGY 1976; 44:139-46

17. Anderson K-E, Edstrom A: Effects of nerve blocking agents on fast axonal transport of proteins in frog sciatic nerves in vitro. Brain Res 1973; 50:125-34

18. Fink BR, Kennedy RD, Hendrickson AE, Middaugh ME: Lidocaine inhibition of rapid axonal transport. ANESTESIOLOGY 1972; 36:422-32

19. Byers MR, Fink BR, Kennedy RD, Middaugh ME, Hendrickson AE: Effects of lidocaine on axonal morphology, microtubules, and rapid transport in rabbit vagus nerve in vitro. J Neurobiol 1973; 4:125-43

20. Lavoie P-A, Khazen T, Filion PR: Mechanisms of the inhibition of fast axonal transport by local anesthetics. Neuropharmacology 1989; 28:175-81

21. Yamamoto H, Fukunaga K, Tanaka E, Miyamoto E: Ca2+- and calmodulin-dependent phosphorylation of microtubule-associated protein 2 and tau factor, and ihibition of microtubule assembly. J Neurochem 1983; 41:1119-25

22. Tashima K, Yamamoto H, Setoyama C, Ono T, Miyamoto E: Overexpression of Ca2+/calmodulin-dependent protein kinase II inhibits neurite outgrowth of PC12 cells. J Neurochem 1996; 66:57-64

23. Masse T, Kelly PT: Overexpression of Ca2+/calmodulin-dependent protein kinase II in PC12 cells alters cell growth, morphology, and nerve growth factor-induced differentiation. J Neurosci 1997; 17:924-31

24. Broughton SJ, Kane NS, Yoder M, Greenspan RJ, Robichon A: Transport of CaM kinase along processes elicited by neuronal contact evokes an inhibition of arborization and outgrowth in D. melanogaster cultured neurons. J Cell Biochem 1996; 62:484-94

25. Hiruma H, Maruyama H, Katakura T, Simada ZB, Nishida S, Hoka S, Takenaka T, Kawakami T: Axonal transport is inhibited by a protein kinase C inhibitor in cultured isolated mouse dorsal root ganglion cells. Brain Res 1999; 826:135-8

26. Gold MS, Reichling DB, Hampl KF, Drasner K, Levine JD: Lidocaine toxicity in primary afferent neurons from the rat. J Phamacol Exp Ther 1998; 285:413-21

27. Oyama Y, Sadoshima J, Tokutomi N, Akaike N: Some properties of inhibitory action of lidocaine on the Ca2+ current of single isolated frog sensory neurons. Brain Res 1988; 442:223-8

28. Garcia-Martin E, Gutierrez-Merino C: Local anesthetics inhibit the Ca2+, Mg2+-ATPase activity of rat brain synaptosomes. J Neurochem 1986; 47:668-72

29. Garcia-Martin E, Gutierrez-Merino C: Modulation of the Ca2+, Mg2+-ATPase activity of synaptosomal plasma membrane by the local anesthetics dibucaine and lidocaine. J Neurochem 1990; 54:1238-46

30. Garcia-Martin E, Gonzalez-Cabanillas S, Gutierrez-Merino C: Modulation of calcium fluxes across synaptosomal plasma membrane by local anesthetics. J Neurochem 1990; 55:370-8

31. Lavoie PA, Bennett G: Accumulation of [3H]fucose-labelled glycoproteins in the Golgi apparatus of dorsal root ganglion neurons during inhibition of fast axonal transport caused by exposure of the ganglion to Co2+-containing or Ca2+-free medium. Neuroscience. 1983; 8:351-62

32. Lavoie PA: Ionic requirements for in vitro retrograde axonal transport of acetylcholinesterase. Neurosci Lett 1982; 33:213-6

33. Lariviere L, Lavoie PA: Calcium requirement for fast axonal transport in frog motoneurons. J Neurochem 1982; 39:882-6

34. Lavoie PA, Bolen F, Hammerschlag R: Divalent cation specificity of the calcium requirement for fast transport of proteins in axons of desheathed nerves. J Neurochem 1979; 32:1745-51

35. Ochs S, Worth RM, Chan SY: Calcium requirement for axoplasmic transport in mammalian nerve. Nature 1977; 270:748-50

36. Lavoie PA, Hammerschlag R, Tjan A: Cobalt ions inhibit fast axonal transport of [3H]glycoproteins but not glycosylation. Brain Res 1978; 149:535-40

37. Dravid AR, Hammerschlag R: Axoplasmic transport of proteins in vitro in primary afferent neurons of frog spinal cord: effect of Ca2+-free incubation conditions. J Neurochem 1975; 24:711-8

38. Gan SD, Fan MM, He GP: The role of microtubules in axoplasmic transport in vivo. Brain Res 1986; 369:75-82

39. Lees GJ: Inhibition of the retrograde axonal transport of dopamine-(-hydroxylase antibodies by the calcium ionophore A23187. Brain Res 1985; 345:62-7

40. Kanje M, Edstrom A, Hanson M: Inhibition of rapid axonal transport in vitro by the ionophores X-537 A and A 23187. Brain Res 1981; 204:43-50

41. Esquerro E, Garcia AG, Sanchez-Garcia P: The effects of the calcium ionophore, A23187, on the axoplasmic transport of dopamine (-hydroxylase. Br J Pharmacol 1980; 70:375-81

42. Kendal WS, Koles ZJ, Smith RS: Oscillatory motion of intra-axonal organelles of Xenopus

laevis following inhibition of their rapid transport. J Physiol 1983; 345:501-13

43. Hiruma H, Maruyama H, Simada ZB, Katakura T, Hoka S, Takenaka T, Kawakami T: Lidocaine inhibits neurite growth in mouse dorsal root ganglion cells in culture. Acta Neurobiol Exp 1999; 59:323-7

Figure 1

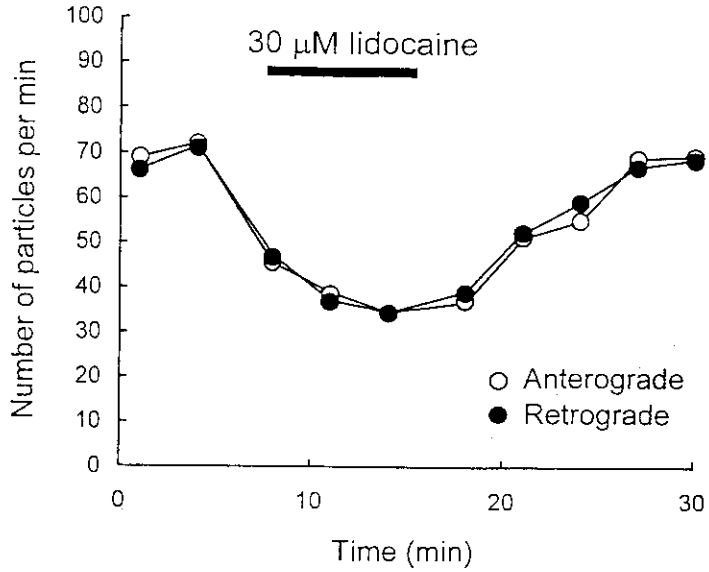


Figure 2

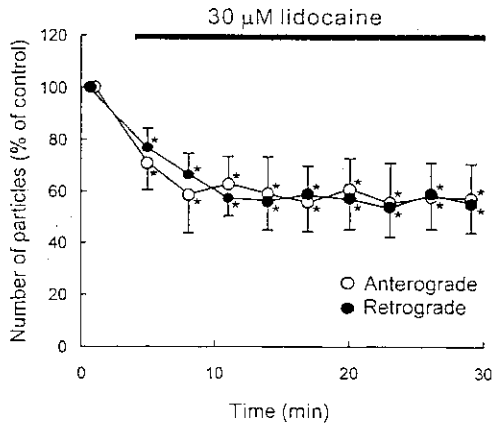


Figure 3

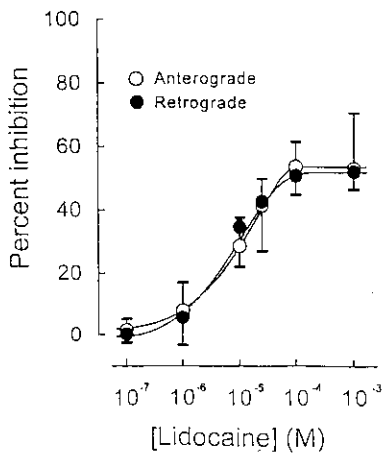


Figure 4

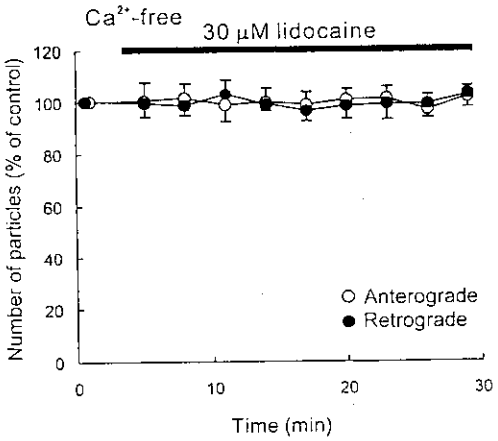


Figure 5

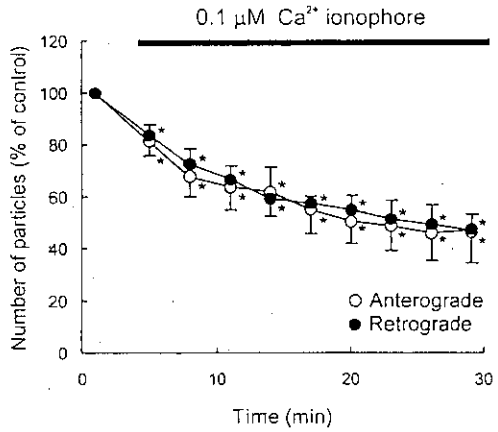


Figure 6

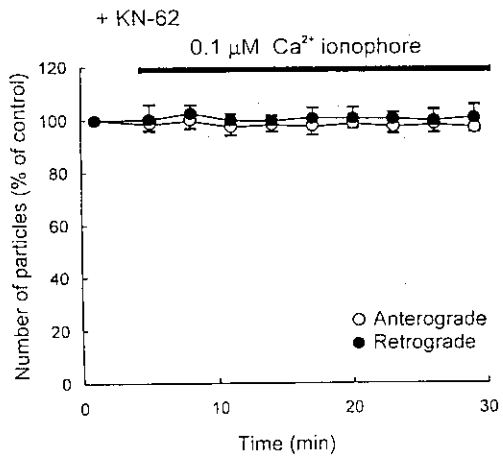
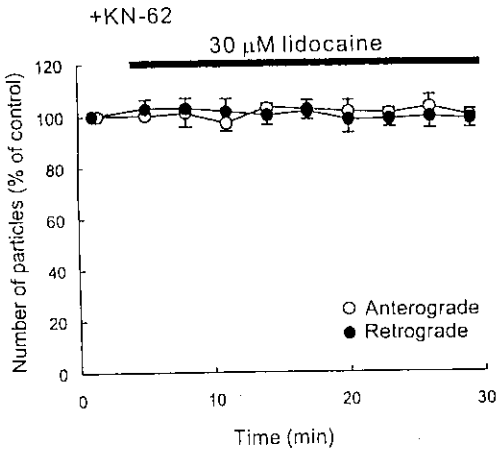


Figure 7



（資料5）　ラット後根神経節細胞の軸索輸送に対する
ペントバルビタールの効果
（北里医学2001　印刷中の原稿）

培養感覚神経におけるpentobarbitalの軸索輸送抑制作用

丸山　宏　　比留間　弘美*　　金井　昭文　　川上　倫*　　外　須美夫

北里大学医学部・麻酔科学、　　*北里大学医学部・生理学

摘要

Barbiturate系の静脈麻酔薬であるpentobarbitalは感覚神経の膜特性やシナプス伝達を修飾することが知られている。本研究では, pentobarbitalが培養感覚神経の軸索輸送に及ぼす影響について検討した。Pentobarbitalを培養マウス感覚神経に投与すると順行性, 逆行性の軸索輸送をともに抑制した。この抑制反応は, GABAA受容体拮抗薬であるbicucullineを前処置した場合や, 無Cl-細胞外液下では認められなかった。Pentobarbitalの細胞膜に対する影響を電気生理学的に検討したところ, pentobarbitalはCl-の透過性を増大することが示された。これらの結果から, pentobarbitalはGABAA受容体を活性化し, Cl-を細胞内に流入させて感覚神経の軸索輸送を抑制することが示唆された。

Key words: pentobarbital, axonal transport, sensory neurons, video-enhanced microscopy, electrophysiology, GABAA receptor, chloride

全身麻酔に使用されるpentobarbitalはbarbiturate系の静脈麻酔薬である。Pentobarbitalは, 従来, 中枢神経系に作用して鎮静効果を示すが, 除痛効果はもたない, あるいは逆に痛覚過敏を起こすとされてきた6,11）。ゆえに, 痛覚受容やその情報伝達を抑制することはないと考えられてきた。しかし, 最近になって, pentobarbital等のbarbiturateは除痛作用をもつということが複数の研究グループによって示唆されてきた3,5,10,15,22,24,32）。これらの報告によると, pentobarbitalは, 一次感覚神経から脊椎後角神経へのシナプス伝達を抑制して除痛効果をもたらすされている22,30,32)。一方, pentobarbitalが末梢感覚神経の膜特性を変えることは多数報告されてきた。Pentobarbitalは, 一次知覚神経の細胞体が存在する脊髄後根神経節 (dorsal root ganglion: DRG) の細胞においてGABA-活性化電流を誘導36)あるいは増強する 8,19,35)ことが示されている。また, この麻酔薬はDRG細胞のcalcium電流を阻害することも示されてきた16,17)。一方, PentobarbitalはGABAA受容体のbarbiturate結合部位に結合し, GABAA受容体と相同であるCl-チャネルを開口させる2)。実際, GABAA受容体はDRG細胞の細胞体および神経線維に密に発現している12,13,18,28）。これらの事実から, pentobarbitalは感覚神経の細胞機能に何らかの影響を及ぼしていることは容易に想像される。

神経の軸索輸送は神経細胞の生存, 機能維持の基盤となる重要な機能である。上述のごとく, pentobarbitalの除痛効果は, 脊髄レベルでのシナプス伝達を抑制して発現することが示されてきた。最近の研究では, 神経因性疼痛のような慢性の痛みの伝達には, 一次感覚神経と脊髄神経間のシナプス形成や, シナプス伝達の増強が関与していることが明らかとなった。実際, pentobarbitalの鎮痛作用も, 急性の痛みには認められず慢性痛においてのみ見られるという15)。これらの事実から類推すると, pentobarbitalが感覚神経のシナプス形成や伝達を修飾している可能性もある。軸索輸送は, 軸索伸展, シナプス形成に必要な物質や伝達物質の輸送も担う神経細胞の基本的機能である。これらの事項から, pentobarbitalは感覚神経の軸索輸送に影響を及ぼす可能性が考えられる。本研究では, この可能性を検証するために, ビデオ増感顕微鏡を用いて, pentobarbitalの培養感覚神経軸索輸送に及ぼす影響について検討した。

材料と方法

細胞培養

雄性成熟マウス（6 週齢）をエーテルにて安楽死させ, 脊髄後根神経節 (DRG) を摘出した。ただちに神経節をHam's F-12培養液中に移した。これらの神経節から神経細胞を単離するために以下に示す酵素処理を行った。すなわち, 0.25% collagenase

（Worthington Biochemical, Freehold, NJ, USA） を含むHam's F-12培養液中で，37℃，90分間，続いて，0.25% trypsin (Sigma Chemical Co., St. Louis, MO, USA) を含む無Ca2+無Mg2+ Hank's平衡塩溶液 (g/l: KCl, 0.4; KH2PO4, 0.06; NaCl, 8; Na2HPO4/7 H2O, 0.09; D-glucose 1; phenol red 0.01; HEPES 3.6; NaOH, 0.3) 中で，37℃，15分間インキュベートした。ただちに0.04% trypsin阻害薬 (Sigma) を添加することによりtrypsin活性を停止した。続いて，10 ml容量の遠心管に入れた30% Percoll 5 mlに，細胞を含む培養液を重層し，遠心 (1500 rpm, 5分間) 後，神経細胞分画を分離した。この分画をHam's F-12培養液で2回洗浄後，火炎処理したピペットを用いて神経節を粉砕することにより細胞を単離した。Poly-lysine (Sigma) 処理したカバーグラス (30×40 mm, 厚さ50 μm) 上に細胞を播種し，10% ウシ胎児血清とpenicillin (100 U/ml)-streptomycin (100 μg/ml) を添加したHam's F-12培養液中にて，37℃，5% CO2 下で48時間培養した。

実験準備

培養48時間後，細胞を培養したカバーグラスを，中央に亀甲型穴 (25×35 mm) をもつ，厚さ0.5 mmのステンレススチール製チャンバー (50×80 mm) の底部に防水テープで貼り付けた。チャンバーの上側を，他のカバーグラス (30×30 mm) で覆った。この際，液を潅流させるために両側に小さな間隙を残した。ただちにHEPES-緩衝生理食塩液 (pH 7.4, 135 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 5.5 mM D-glucose) をチャンバー内に入れた。チャンバーをZeiss Axiomat倒立型顕微鏡 (Carl Zeiss, Oberkochen, Germany) のステージ上に固定した。ステージを温度コントローラーにより37℃に維持した。試薬を投与する際には，パスツールピペットを用いて試薬溶液 (3 ml) を一方の間隙から注入し，反対側の間隙から流出した液は，吸引ポンプを用いて除去した。

薬物

Pentobarbital sodium, (-) bicuculline methiodide (両方ともSigma, St. Louis, MO, USA) はHEPES-緩衝生理食塩液で溶解して用いた。Pentobarbitalが培養神経細胞のCl-電流を増強させる濃度のEC50値がラットで84 ± 3 μM, マウスで106 ± 6 μMであることから1)、pentobarbitalの使用濃度は100 μMとした。また、bicucullineの使用濃度は、培養神経細胞でGABA性Cl-電流を阻止する濃度である100 μMとした35)。Cl-の関与を調べるために，低Cl-溶液 (pH 7.3, 67.5 mM Na2SO4, 2.5 mM K2SO4, 1 mM CaCl2, 1 mM MgSO4, 10 mM HEPES, 5.5 mM D-glucose) を準備した。

ビデオ増感顕微鏡法

倒立型微分干渉顕微鏡によって得られるノマルスキー像を，アナログビデオカメラ (Harpicon, Hamamatsu Photonics, Hamamatsu, Japan) で取り込み，アナログ電気信号 (一次元データ) に変換した。アナログ信号を，イメージプロセッサー (DVS-20; Hamamatsu Photonics) によって処理し，デジタル変換，デジタル増強及び画像改善処理 (積算処理，背景減算，コントラスト増強) を行った。この像を，連続的にリアルタイムでビデオモニター (C1864; Hamamatsu Photonics) 上に表示し，ビデオレコーダー (PVW-2800; Sony, Tokyo, Japan) で録画，保存した。

軸索輸送の解析

軸索輸送の解析は，粒子 (直径？50 nm) の数を計測することによって行った。録画したビデオ像を再生し，軸索の長軸に対して垂直の線をビデオモニター上に描出し，その線を通過する粒子数を計測した。計測は，試薬投与前後のそれぞれの期間について3分毎に2分間行い，粒子数/分を計算した。データは平均値 (mean) ± 標準偏差 (standard deviation, S.D.) で示した。結果の統計学的有意性はStudent-t 検定を用いて求めた。

電気生理学的記録

膜電位をwhole-cell記録法で記録した (37℃)。Whole-cell 記録は，150 mM K-gluconate (Sigma), 1 mM MgCl2, 10 mM HEPESと1 mM EGTAで組成される溶液 (pH 7.2) で満たされたパッチパイペット (抵抗: 1-5 MΩ) を用いて行った。実験記録用の細胞外液として上記のHEPES-緩衝生理食塩液，あるいは，低Cl-液を用いた。薬物は，溶液槽に投与した。電気信号をpatch-clamp L/M-EPC7 (List Electronic, Darmastadt, Germany) で増幅し，オシロスコープとチャートレコーダー上でモニターした。

結果

マウスの脊髄後根神経節細胞を48時間培養後，他の細胞との接触がないものを実験

に供した。コントロール細胞外液 (HEPES-緩衝溶液) 中では，順行性，逆行性の輸送粒子数/分の平均値はそれぞれ，59.4 ± 12.2 (mean ± S.D., n=16)，59.4 ± 14.6 (n=16) であった。

Pentobarbitalの軸索輸送に及ぼす影響

Pentobarbital (100 μM) を短時間 (10分間) 投与したところ，順行性，逆行性ともに輸送される粒子数 (/分) はただちに減少した。HEPES-緩衝生理食塩液で洗い流すと，粒子数は元のレベルに戻ったことから，この反応は可逆的であると考えられた (Fig. 1)。

Pentobarbital (100 μM) を持続投与 (26分間) したところ，投与2分後には順行性，逆行性の輸送粒子数は有意に減少し，投与17分後には最小値 (投与前の約50%) に達した。この値は投与期間中戻ることなく維持された (Fig. 2)。このような反応は，実験を行った5例すべての細胞にみられた。

GABAA受容体拮抗薬存在下におけるpentobarbitalの効果

PentobarbitalはGABAA受容体のbarbiturate結合部位に結合する12,13,18,28)。また，感覚神経においてGABAA受容体とそのmRNAが発現していることが明らかになっている12,13,18,28)。そこで，pentobarbitalの軸索輸送抑制反応におけるGABAA受容体の関与を調べる目的で，GABAA受容体拮抗薬 (antagonist) の存在下でpentobarbitalを投与し軸索輸送の反応を観察した。GABAA受容体拮抗薬である bicuculline (100 μM) の存在下ではpentobarbitalの抑制反応は検出されなかった (Fig.3)。同様の反応は，実験を行った5例すべての細胞にみられた。

低Cl-細胞外液下におけるpentobarbitalの効果

GABAA受容体はCl-チャネルを内蔵しており，活性化によりCl-チャネルが開口し，Cl-は細胞内外の電気的勾配と濃度勾配の差に従って流出入する。用いた細胞では，Cl-が細胞内に流入して軸索輸送が抑制される可能性がある。このことを検証するために，低Cl-細胞外液下でpentobarbitalの効果を調べた。低Cl-細胞外液中では，pentobarbitalの軸索輸送抑制反応はみられなかった。このような反応は，実験を行った5例すべての細胞にみられた (Fig.4)。

Pentobarbitalによる培養DRG細胞膜の応答

Pentobarbitalが培養DRG細胞膜のCl-チャネルを開口させることを確かめるために，whole-cell 記録を行うことによって，pentobarbitalによる膜特性の変化について調べた。Cl-濃度が 2 mMのパッチパイペット内液で細胞内を還流しながらpentobarbitalを投与すると，細胞膜は過分極応答を示した (Fig.5A)。同時に，パルス状に一定電流を与え，それに応答する膜電位の変化を観察することにより，膜抵抗の変化を調べた。Pentobarbitalを投与するとパルス状の一定電流に応答する膜電位の反応は小さくなった (Fig.5)。すなわち，pentobarbitalにより膜抵抗 (入力抵抗) が減少した。さらに、与えた電流と応答する電圧の関係から，pentobarbitalを投与した際の逆転電位は-73 mVであった。この実験条件下では，細胞外のCl-濃度は144 mM (HEPES-緩衝溶液のCl-濃度)であり，細胞内は2 mMのCl-液で還流している。還流が不完全であることを考えて，細胞内のCl-濃度を8 mMと仮定した。このCl-濃度条件からNernstの式を用いてCl-の平衡電位を求めてみた。

Nernst の式

$$E_{Cl} = RT/FZ_{Cl} \ln [Cl^-]_o/[Cl^-]_i$$

E_{Cl}: Cl-の平衡電位, R: 気体定数, T: 絶対温度, F: Faraday 定数 (1 モルの1価イオンのもつ電荷量), Z_{Cl}: Cl-の原子価 (-1), $[Cl^-]_o$: Cl-の細胞外濃度, $[Cl^-]_i$: Cl-の細胞内濃度

すなわち,

$$E_{Cl} = 58 \log [Cl^-]_i/[Cl^-]_o \quad (37^\circ C)$$

となる。

ここで, $[Cl^-]_i = 8$ および $[Cl^-]_o = 144$を代入すると, $E_{Cl} = -72.8$ mVとなり，実験で観察されたpentobarbitalの逆転電位とほぼ一致する。

次に，細胞外液を2 mMのCl-溶液に置き換えてから，pentobarbitalを投与すると，細胞は脱分極応答を示した (Fig.5B)。この際にも，膜抵抗は減少し，逆転電位は+35 mVを示した。この場合にも，Nernstの式を適用してみる。すなわち，細胞外Cl-濃度は2 mMで，細胞内Cl-濃度を8 mMと仮定すると，理論値は+34.9 mVとなり，実際の測定値とほぼ一

致する。つまり,細胞内Cl-濃度が8 mMであるとすると,細胞外液のCl-濃度を変えても,pentobarbitalの逆転電位はCl-の平衡電位と一致することを示している。

考察

以上の結果から，pentobarbitalは培養マウスDRG細胞において順行性，逆行性の軸索輸送を抑制することが明らかになった。この反応はpentobarbital投与後数分以内に検出された。この反応はpentobarbital投与期間中には戻ることはないが,pentobarbitalを洗い流すことによって元に戻る可逆的な反応であると考えられる。

本研究ではさらにpentobarbitalによる軸索輸送抑制作用の機序についても明らかにした。PentobarbitalはGABAA受容体のbarbiturate結合部位に結合することが知られている。GABAA受容体拮抗薬であるbicucullineを前処置した細胞では，pentobarbitalの軸索輸送抑制効果は認められなかった。従って,pentobarbitalの軸索輸送抑制作用はGABAA受容体を活性化して発現することが確かめられた。さらに,pentobarbitalはGABAA受容体と相同であるCl-チャネルを開口させ,Cl-がCl-チャネルを通って細胞内外に出入りすると推測される。以前の研究において,細胞内のCl-濃度の増加は感覚神経の軸索輸送を抑制させることが示されている21)。このことからも,pentobarbitalを投与すると,Cl-が細胞内に流入して軸索輸送を抑制する可能性がある。このことを確かめるために,細胞外液を低Cl-にし,pentobarbitalの軸索輸送抑制作用が阻止されるか否か調べた。その結果,低Cl-細胞外液中ではpentobarbitalは軸索輸送に影響を与えないことが明らかとなった。従って,pentobarbitalはGABAA受容体を活性化し,Cl-を細胞内に流入させて軸索輸送を抑制することが示唆された。

さらに本研究では,電気生理学的にpentobarbitalによって変化する膜特性について調べ,上に述べたpentobarbital作用の細胞膜機序について確かめた。低Cl-濃度のパッチパイペット内液を用いて,細胞内を低Cl-液に置換した場合,pentobarbitalは培養DRG細胞膜を過分極させる。また,pentobarbitalは膜抵抗を減少させ,pentobarbitalの逆転電位はCl-の平衡電位とほぼ等しかったことから,pentobarbitalはCl-チャネルを開口させることが示された。つづいて,細胞外液も低Cl-液にした場合,細胞はpentobarbitalにより脱分極応答を示した。この条件で求められるpentobarbitalの逆転電位もCl-の平衡電位と等しかった。これらの電気生理学的実験結果は,pentobarbitalの軸索輸送抑制作用はCl-の透過性増大によってもたらされるという考えを支持するものである。

しかし,このことに関し,以前の電気生理学的研究と照らし合わせると疑問が生じた。それは,感覚神経細胞の場合,Cl-チャネルが開口すると本当にCl-が細胞内に流入するかという問題である。電気生理学的にGABAやpentobarbitalを含めたGABAA受容体作動薬は,感覚神経細胞等の末梢神経細胞や若年期の中枢神経細胞を脱分極させる9)。この現象は,KClではなくK-acetateやK-sulfateで内部を満たされた細胞内記録用電極を用いても,すなわち,細胞内Cl-の増加が起こらない条件においても,起こる。この理由は,これらの細胞は,内向きCl-トランスポータの働きによって細胞内Cl-濃度が大きいために,Cl-の平衡電位が静止膜電位より高いからである,と説明されていた4,14,26)。この説に従えば,GABAA受容体が活性化するとCl-は細胞外に流出することになる。従って,この説と照らし合わせれば,本研究結果は矛盾することになる。

しかし最近,GABAA受容体はCl-のみならずHCO3-等の陰イオンを通すことが明らかになってきた4)。従って,GABAA受容体作用による膜電位の脱分極応答は,Cl-が細胞内に流入すると同時にHCO3-が細胞外に流出することによって起こるという提唱がなされた。すなわち,HCO3-の平衡電位は静止膜電位より非常に高いので,脱分極するという27,31)。この説は,GABAA受容体活性化によって膜が脱分極するにもかかわらずCl-が流入することを意味し,本研究結果を支持するものである。

さらにごく最近,細胞内Cl-濃度のイメージング法を用いた研究によって,P19-NというGABAA受容体の刺激で脱分極する細胞においても,GABAA受容体作動薬により細胞内Cl-濃度が上昇することが判明した7)。従って,感覚神経細胞においても,GABAA受容体刺激で脱分極するにもかかわらずCl-が細胞内に流入する可能性があることが考えられた。

現在のところ,細胞内に上昇したCl-がさらにどのようにして軸索輸送を抑制するのか不明である。しかし,細胞内のCl-濃度の上昇は細胞毒性を発揮し,細胞膨潤を引き起こし,ひいては細胞死に至ることが示唆されている29)。さらに最近の研究では,細胞内のCl-はG蛋白活性を変えることが示されている20,23,25)。軸索輸送は,他の多くの細胞機能と同様,G蛋白機能によって調節を受けている。G蛋白を阻害する百日咳毒素は,アセチルコリンで誘導される軸索輸送抑制反応を阻止することが報告されている33,34)。従って,細胞内に増加したCl-は軸索輸送を修飾するG蛋白活性を変化させて軸索輸送を抑制する可能性が考えられる。

結語

本研究では, pentobarbitalが感覚神経の軸索輸送を抑制することを提示した。その作用機序として, GABAA受容体を介してCl-の細胞内流入によって誘導されることが示唆された。本研究結果から, pentobarbitalの軸索輸送抑制作用が, 感覚神経における軸索伸展やシナプス形成あるいはシナプス伝達を抑制させて除痛作用へと導く可能性も考えられる。

文献

1) Aguayo, L.G., Pancetti, F.C., Klein, R.L. & Harris,R.A.: Differential effects of GABAergic ligands in mouse and rat hippocampal neurons. Brain Res., 647: 97~105, 1994.

2) Barnard, E.A., Skolnick, P., Olsen, R.W., Mohler, H., Sieghart, W., Biggio, G., Braestrup, C., Bateson, A.N. & Langer, S.Z.: International union of pharmacology. XV. Subtypes of γ -aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function. Pharmacol. Rev., 50: 291~313, 1998.

3) Bertens, A.P.: The effects of two barbiturates, pentobarbital and thiopental, on the nociceptive electrically and nociceptive mechanically evoked potentials in dogs. Electromyogr. Clin. Neurophysiol., 26:241~262, 1986.

4) Bormann, J.: Electrophysiology of GABAA and GABAB receptor subtypes. Trends Neurosci., 11: 112~116, 1988.

5) Brian, A., Jewett, B.A., Lisa, M., Gibbs, B.M., Tarasiuk, A. & Kendig, J.J.: Propofol and barbiturate depression of spinal nociceptive neurotransmission. Anesthesiology, 77: 1148~1154, 1992.

6) Carlsson, K.H. & Jurna, I.: Interaction of pentobarbital and morphine in the tail-flick test performed on rats: synergism at the spinal and antagonism at the supraspinal level. Neurosci. Lett., 71: 356~360, 1986.

7) Chistina Grobin, A., Inglefield, J.R., Schwartz-Bloom, R.D., Devaud, L.L. & Morrow, A.L.: Fluorescence imaging of GABAA receptor-mediated intracellular [Cl-] in P19-N cells reveals unique pharmacological properties. Brain Res., 827: 1~11, 1999.

8) Connors, B.W.: A comparison of the effects of pentobarbital and diphenylhydantoin on the GABA sensitivity and excitability of adult sensory ganglion cells. Brain Res., 207: 357-369, 1981.

9) Desarmenien, M., Santagelo, F., Linck, G., Headley, P. M. & Feltz, P.: Physiological study of amino acid uptake and receptor desensitization: The GABA system in dorsal root ganglia. In "Amino Acid Neurotransmitters" Ed. by F.V. DeFeudis and P. Mandel, pp. 309~319, Raven Press, New York, 1981.

10) Doi, T. & Jurna, I.: Intrathecal pentobarbital prevents naloxone-induced facilitation of the tail-flick response in the rat. Neurosci. Lett., 32: 81~84, 1982.

11) Dundee, J.W.: Alteration in response to somatic pain associated with anaesthesia. II: The effect of thiopentone and pentobarbiyone. Br. J. Anaesth., 32: 407~414, 1960.

12) Fukuoka, T., Tokunaga, A., Kondo, E., Miki, K., Tachibana, T. & Noguchi, K.: Change in mRNAs for neuropeptides and the GABA(A) receptor in dorsal root ganglion neurons in a rat experimental neuropathic pain model. Pain, 78: 13~26, 1998.

13) Furuyama, T., Sato, M., Sato, K. Araki, T., Inagaki, S., Takagi, H. & Tohyama, M.: Co-expression of glycine receptor beta subunit and GABAA receptor gamma subunit mRNA in the rat dorsal root ganglion cells. Brain Res. Mol. Brain Res., 12: 335~338, 1992.

14) Gallagher, J.P., Higashi, H. & Nishi, S.: Characterization and ionic basis of GABA-

induced depolarizations recorded in vitro from cat primary afferent neurones. J. Physiol., 275: 263~282, 1978.

15) Goto, T., Marota, J.A. & Grosby, G.: Pentobarbitone, but not propofol, produces pre-emptive analgesia in the rat formalin model. Br. J. Anaesth., 72: 662~667, 1994.

16) Gross, R.A. & Macdonald, R.L.: Differential actions of pentobarbitone on calcium current components of mouse sensory neurones in culture. J. Physiol., 405: 187~203, 1988.

17) Gross, R.A. & Macdonald, R.L.: Barbiturates and nifedipine have different and selective effects on calcium currents of mouse DRG neurons in culture: a possible basis for differing clinical actions. Neurology, 38: 443~451, 1988.

18) Gutierrez, A., Khan, Z.U. & De Blas, A.L.: Immunocytochemical localization of the alpha 6 subunit of the gamma-aminobutyric acidA receptor in the rat nervous system. J. Comp. Neurol., 365: 504~510, 1996.

19) Headley, P.M., Desarmenien, M., Santangelo, F. & Feltz, P.: Direct action of pentobarbitone in potentiating the responses to GABA of rat dorsal root ganglion neurones in vitro. Neurosci. Lett., 24: 273~280, 1981.

20) Higashijima, T., Ferguson, K.M. & Sternweis, P.C.: Regulation of hormone-sensitive GTP-dependent regulatory proteins by chloride. J. Biol. Chem., 262: 3597~3602, 1987.

21) Hiruma, H., Nishida, S., Katakura, T., Kusakabe, T., Takenaka, T. & Kawakami, T.: Extracellular potassium rapidly inhibits axonal transport of particles in cultured mouse dorsal root ganglion neurites. J. Neurobiol., 38: 225~233, 1999.

22) Jewett, B.A., Gibbs, L.M., Tarasiuk, A. & Kendig, J.J.: Propofol and barbiturate depression of spinal nociceptive neurotransmission. Anesthesiology. 77: 1148~1154, 1992.

23) Lenz, R.A., Pitler, T.A. & Alger, B.E.: High intracellular Cl- concentrations depress G-protein-modulated ionic conductances. J. Neurosci., 17: 6133-6141, 1997.

24) McCarthy, M.M., Beyer, C. & Komisaruk, B.R.: Barbiturate-induced analgesia: Permissive role of a GABA-A agonist. Pharmacol. Biochem. Behav., 32: 897~900, 1989.

25) Nakajima, T., Sugimoto, T. & Kurachi, Y.: Effects of anions on the G protein-mediated activation of the muscarinic K+ channel in the cardiac atrial cell membrane. Intracellular chloride inhibition of the GTPase activity of GK. J. Gen. Physiol., 99: 665~682, 1992.

26) Nishi, S., Minota, S. & Karczmar, A.G.: Primary afferent neurones: the ionic mechanism of GABA-mediated depolarization. Neuropharmacol., 13: 215~219, 1974.

27) Perkins, K.L. & Wong, R.K.: Ionic basis of the postsynaptic depolarizing GABA response in hippocampal pyramidal cells. J. Neurophysiol., 76: 3886~3894, 1996.

28) Persohn, E., Malherbe, P. & Richards, J.G. :In situ hybridization histochemistry reveals a diversity of GABAA receptor subunit mRNAs in neurons of the rat spinal cord and dorsal root ganglia. Neuroscience, 42: 497~507, 1991.

29) Rothman, S.M.: The neurotoxicity of excitatory amino acids is produced by passive chloride influx. J. Neurosci., 5: 1483~1489, 1985.

30) Sandkuhler, J., Fu, Q., Helmchen, C. & Zimmermann, M.: Pentobarbital, in subanesthetic doses, depresses spinal transmission of nociceptive information but does not affect stimulation-produced descending inhibition in the cat. Pain, 31: 381~390, 1987.

31) Staley, K.J., Soldo, B.L. & Proctor, W.R.: Ionic mechanisms of neuronal excitation by inhibitory GABAA receptors. Science, 269: 977~981, 1995.

32) Stein, C., Morgen, M.M. & Liebeskind, J.C.: Barbiturate-induced inhibition of a spinal nociceptive reflex: Role of GABA mechanisms and descending modulation. Brain Res., 407: 307~311, 1987.

33) Takenaka, T., Kawakami, T., Hikawa, N., Bandou, Y. & Gotoh, H.: Effect of neurotransmitters on axoplasmic transport: acetylcholine effect on superior cervical ganglion cells. Brain Res., 588: 212~216, 1992.

34) Takenaka, T., Kawakami, T., Hori, H. & Bandou, Y.: Effect of neurotransmitters on axoplasmic transport: how adrenaline affects superior cervical ganglion cells. Brain Res., 643: 81~85, 1994.

35) Valeyev, A.Y., Hackman, J.C., Holohean, A.M., Wood, P.M., Katz, J.L. & Davidoff, R.A.: GABA-Induced Cl- current in cultured embryonic human dorsal root ganglion neurons. J. Neurophysiol., 82: 1~9, 1999.

36) Yakushiji, T., Oyama, Y. & Akaike, N.: Comparative study on barbiturates using isolated single neurons: GABA-mimetic action and augmentatory action on GABA response. Brain Res., 488: 357~360, 1989.

Inhibitory Effects of Pentobarbital on Axonal Transport in Sensory Neurons

Hiroshi Maruyama, Hiromi Hiruma*, Akifumi Kanai, Tadashi Kawakami*,
Sumio Hoka

Departments of Anesthesiology and Physiology*
Kitasato University School of Medicine

The effects of pentobarbital, a general anesthetic, on axonal transport in cultured mouse sensory neurons were examined using video-enhanced microscopy. Application of pentobarbital decreased the number of particles being transported in anterograde and retrograde directions. The inhibition of axonal transport to pentobarbital was prevented by bicuculline, a GABAA receptor antagonist and by removal of Cl- from extracellular medium. Electrophysiological experiments revealed that pentobarbital increased Cl- permeability in cultured sensory neurons. These results suggest that pentobarbital inhibits axonal transport in sensory neurons via Cl- influx through Cl- channels that are homogenous to GABAA receptors.

図の説明

Fig. 1. Effects of a transient application (10 min) of pentobarbital on axonal transport in cultured mouse dorsal root ganglion cells.
The graph plots the changes in numbers of transported particles in anterograde and retrograde directions induced by 100 μ M pentobarbital.

Fig. 2. Effects of a prolonged application (26 min) of pentobarbital on axonal transport.
The graph plots the percent changes in the number of transported particles evoked by 100 μ M pentobarbital. Each point illustrates the mean (±S.D.) of the values observed in five dorsal root ganglion cells.
*$p<0.05$, **$p<0.005$.

Fig. 3. Effect of pentobarbital on axonal transport in the presence of bicuculline.
The graph plots the percent changes in the number of transported particles after the addition of 100 μ M pentobarbital in the presence of 100 μ M bicuculline, a GABAA receptor antagonist. Each point illustrates the mean(±S.D.) of the values observed in five dorsal root ganglion cells.

Fig. 4. Effect of pentobarbital on axonal transport in low Cl-- extracellular medium.
The graph plots the percent changes in the number of transported particles after the addition of 100 μ M pentobarbital in low Cl-- extracellular medium. Each point illustrates the mean(±

S.D.) of the values observed in five dorsal root ganglion cells.

Fig. 5 Effects of pentobarbital on membrane potential and resistance in cultured mouse dorsal root ganglion cells.
Whole-cell current-clamp recordings were obtained from cultured mouse dorsal root ganglion cells. Low Cl- (2 mM)-patch pipettes (pH 7.2: 150 mM K-gluconate; 1 mM MgCl2; 1 mM EGTA; 10 mM HEPES) were used. Recordings were made in extracellular medium containing 144 mM Cl- or 2 mM Cl-. Traces show the voltage responses to 100 (M pentobarbital. Downward deflections are electrotonic voltage responses to the application of fixed 100 pA-current pulses.

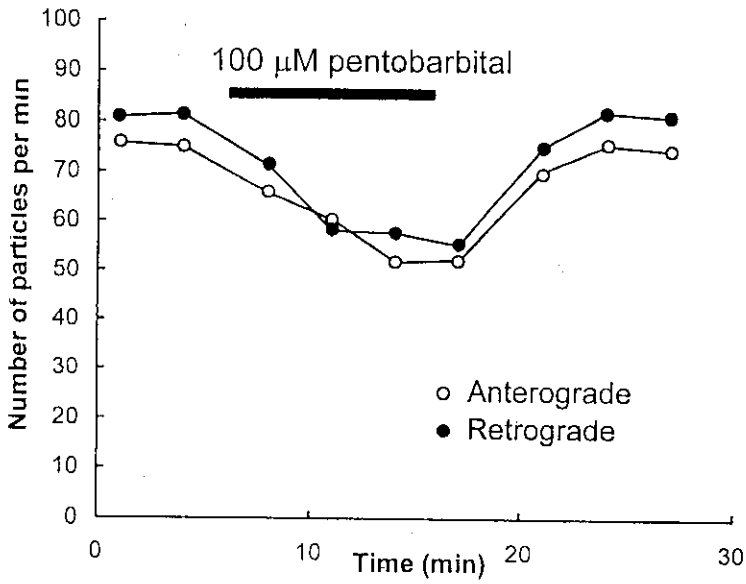


Fig. 1 Effects of a transient application (10 min) of pentobarbital on axonal transport in cultured mouse dorsal root ganglion cells.
The graph plots the changes in numbers of transported particles in anterograde and retrograde directions induced by 100 μM pentobarbital.

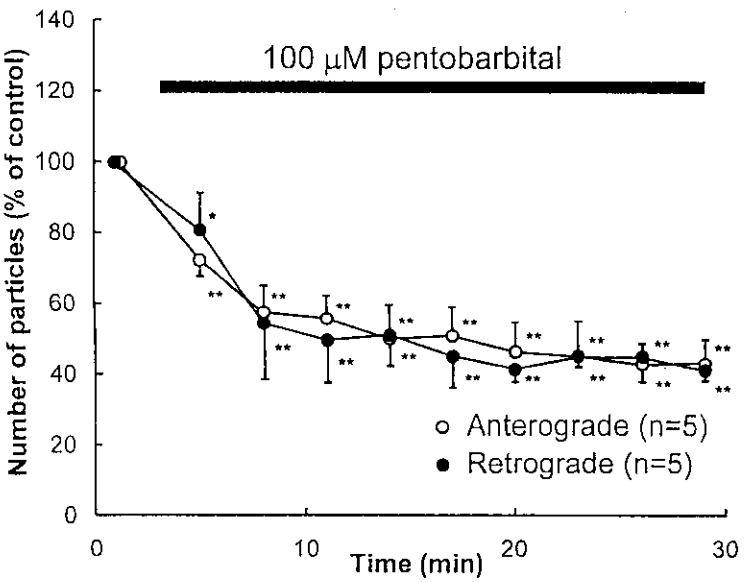


Fig. 2 Effects of a prolonged application (26 min) of pentobarbital on axonal transport.
The graph plots the percent changes in the numbers of transported particles evoked by 100 μM pentobarbital. Each point illustrates the mean (±SD) of the values observed in five dorsal root ganglion cells.
*p<0.05, **p<0.005

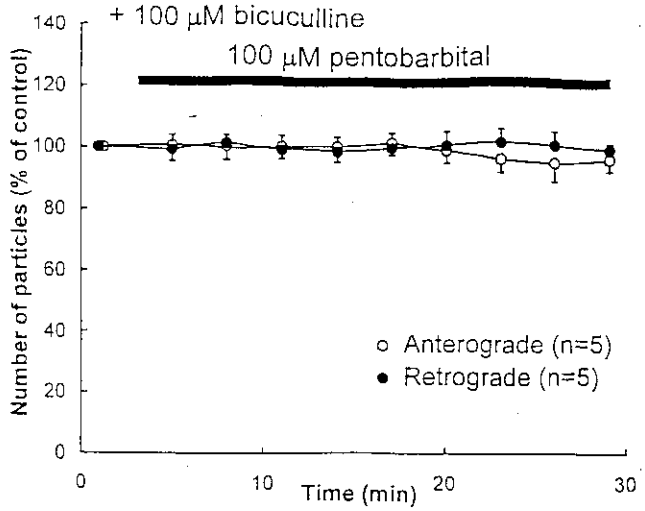


Fig. 3 Effects of pentobarbital on axonal transport in the presence of bicuculline.
The graph plots the percent changes in the numbers of transported particles after the addition of 100 μM pentobarbital in the presence of 100 μM bicuculline, a $GABA_A$ receptor antagonist. Each point illustrates the mean (±SD) of the values observed in five dorsal root ganglion cells.

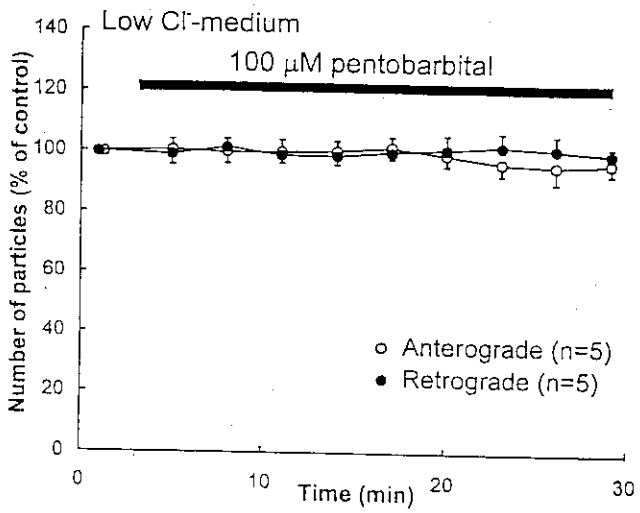


Fig. 4 Effect of pentobarbital on axonal transport in low Cl^--extracellular medium.
The graph plots the percent changes in the numbers of transported particles after the addition of 100 μM pentobarbital in low Cl^--extracellular medium. Each point illustrates in the mean (±SD) of the values observed in five dorsal root ganglion cells.

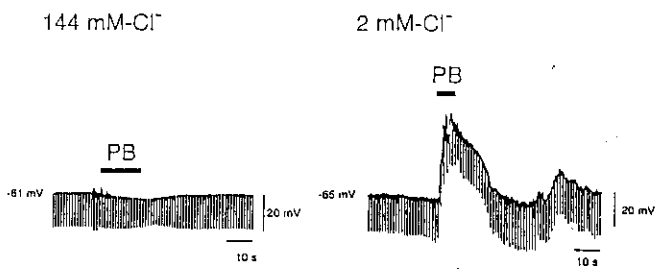


Fig. 5 Effects of pentobarbital on membrane potential and resistance in cultured mouse dorsal root ganglion cells.
Whole-cell current-clamp recordings were obtained from cultured mouse dorsal root ganglion cells. Low Cl^- (2 mM)-patch pipettes (pH 7.2: 150 mM K-gluconate; 1 mM $MgCl_2$; 1 mM EGTA; 10 mM HEPES) were used. Recordings were made in extracellular medium containing 144 mM Cl^- or 2 mM Cl^-. Trances show the voltage responses to 100 μM pentobarbital. Downward deflections are electrotonic voltage responses to the application of fixed 100 pA-current pulses.

研究報告書

厚生科学研究費補助金（長寿科学総合研究事業）

分担研究報告書-1

高齢者の疼痛緩和に関する研究

分担研究者　奥富俊之　北里大学医学部麻酔科講師

研究要旨; 研究要旨;麻酔法として全身麻酔の侵襲を避けるために、あるいは術後の痛みを軽減する目的で硬膜外麻酔が用いられるが、その際の薬物投与の量と、同時に使用される生理食塩水の量と時間の関係を検討した結果、生理食塩水を多く用いると鎮痛効果と冷覚消失に差が生じることが明らかになり、分離麻酔効果が出現することが示唆された。

A.研究目的
高齢者の手術時の疼痛を軽減するための、硬膜外麻酔はよく用いられる麻酔法である。硬膜外麻酔を施行する時には、圧消失法を用いる。その際、生理食塩水を使用することが多い。また、カテーテル挿入が上手く行かないときには、結果的に生理食塩水を多く使用することになる。この生理食塩水の投与量と麻酔効果をみた研究は少ない。本研究では、硬膜外麻酔に用いられる生理食塩水の量と投与時間により硬膜外麻酔の効果が差があるかを検討した。

B.研究方法
北里大学東病院で手術をうける患者に硬膜外麻酔を施行し、圧消失法に用いる生理食塩水の量を3段階に分けて、メインドーズのメピバカインの麻酔効果がどのように修飾されるかを検討した。対象は152人のASAI-III の患者で上腹部手術を受ける患者であった。硬膜外麻酔を胸椎7-8番目から施行した。患者を生理食塩水を1ml,5ml, 10ml投与し、その後、1%メピバカイン、1.5%メピバカインを投与した。皮膚の冷覚消失法とピンプリックによる疼痛刺激法で麻酔効果を測定した。

C.研究結果
生理食塩水を 5ml, 10ml 投与したグループでは、1.5%メピバカインを投与したあとの冷覚消失範囲が、生理食塩水を1ml使用したグループより有意に大きかった。しかし、ピンプリックによる疼痛刺激での麻酔範囲に有意な差は認められなかった。このことから、硬膜外麻酔に用いられる生理食塩水の量をふやしていくと、ピンプリック方式で行う痛み刺激に反応する領域と冷覚刺激に反応する領域に違いが生じることが分かった。

D.考察
硬膜外麻酔時に生理食塩水を用いれば用いるほど、鎮痛領域と冷覚消失領域に違いが生じる。このことは、脊髄麻酔の時によく認められる分離麻酔に相当する。よって、圧消失法で生理食塩水を用いるときにはこの点に注意が必要である。この分離麻酔の機序は不明であるが、

E.結論
硬膜外麻酔時の圧消失法に生理食塩水を用いれば、分離麻酔を来す可能性がある。

F.健康危険情報
なし

G.研究発表
1.論文発表
○Okutomi T. Mochizuki J. Amano K. Hoka S. Effect of epidural epinephrine infusion with bupivacaine on labor pain and mother-fetus outcome in humans.
Regional Anesthesia & Pain Medicine. 25:228-34, 2000
○Morishima H ,Okutomi T ,R.A. Whittington , T.B. Cooper ;Dose-related differences in the distribution of cocaine in the maternal-fetoplaceutal eonpartinents in rats. Neurotoxicol Teratol 22(6);837-844 ,2000
○Okutomi T , Yano K ,Morishima H ;Effect of standard diluted epinephrine infusion on epidural anesthesia in labor.Reg Anesth Pain Med 25(5);529-534 ,2000