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

Akiyumi Kanai, M.D., *  Hiromi Hiruma, M.D., †  Takashi Katakuraki, B.Sc., ‡  Sumi Sase, M.D., *  Tadashi Kawakami, M.D., §  Sumio Hoka, M.D. ||

**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 current study, the authors investigated the effects of the analgesic agent lidocaine on axonal transport in neurites of cultured mouse dorsal root ganglion neurons. In relation to their effects, the effects of lidocaine on the growth rate of the neurite were also examined.

**Methods:** Isolated mouse dorsal root ganglion 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 during control conditions and in the presence of lidocaine.

**Results:** Application of 30 μM lidocaine immediately reduced the number of particles transported in anterograde and retrograde axonal directions. These effects were persistently observed during the application (26 min) and were reversed by lidocaine washout. The inhibitory effect was dose-dependent at concentrations from 0.1 to 1,000 μM (IC₅₀ = 10 μM). In Ca²⁺/-free extracellular medium, lidocaine failed to inhibit axonal transport. Calcium ionophore A23187 (0.1 μM) reduced axonal transport in both directions. The inhibitory effects of lidocaine and A23187 were abrogated by 10 μM KN-62, a Ca²⁺-calmodulin-dependent protein kinase II inhibitor. Application of such low-concentration lidocaine (30 μM) 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 calmodulin-dependent protein kinase II.

**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 weeks old) were killed with ether, and the dorsal root ganglia were removed. The ganglia were immersed immediately in Ham’s F-12 culture medium (Gibco BRL, Grand Island, NY) and incubated for 90 min at 37°C in Ham’s 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 Hank’s balanced salt solution (0.4 g/l KCl, 0.06 g/l KH₂PO₄, 8 g/l NaCl, 0.09 g/l Na₂PO₄/7H₂O, 1 g/l glucose, 0.01 g/l phenol red, 3.6 g/l HEPES, and 0.3 g/l NaOH) 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 Ham’s F-12 medium, the ganglia were triturated using fire-polished pipettes (ID = 0.2–0.5 mm). The isolated cells were plated onto polylysine-coated
coverglasses and cultured for 48 h at 37°C in Ham's F-12 medium containing 10% fetal bovine serum and penicillin (100 units/ml)–streptomycin (100 μg/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 × 80 mm) with a lozenge-shaped hole (25 × 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 64× 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 (Sigma) and then diluted with aqueous solution. The dimethyl sulfoxide concentration was 0.01% and, at this concentration, had no effect on axonal transport and neurite growth.

**Observation of Axonal Transport**

Nomarski images of neurites (length ≥ 100 μm, width ≥ 1 μm) obtained by inverted microscopy were transformed into video images with enhanced contrast using a video camera (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, Japan). 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 previously. The chamber was filled with PSS and maintained at 37°C. Length of neurites (width ≥ 1.0 μm) was measured just before and 30 min after treatment with drugs under video-enhanced microscopy at ×3,000 magnification. PSS was applied in control cells.

**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 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 paired t test. Neurite growth rates are expressed as mean ± SD. The statistical significance of difference in the growth rate between control (nontreated cells) and treated cells was determined by Student 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 ranged from 100 to 360 μm.

**Effects of Low-concentration Lidocaine on Axonal Transport**

Application of lidocaine at low concentration (30 μM) 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 μM) 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 and 1,000 μM indicated that the effect of the drug on axonal transport was dose-dependent (fig. 3). The median inhibitory concentration (IC₅₀) was 10 μM 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 μM lidocaine failed to decrease the number of parti-
cles in either anterograde or retrograde direction (video 1B, fig. 2B). These results suggest that extracellular Ca\(^{2+}\) is required for the inhibition of axonal transport induced by lidocaine.

### Effects of Activation of Calmodulin-dependent Protein Kinase II on Axonal Transport

Failure of lidocaine to inhibit axonal transport in the absence of extracellular Ca\(^{2+}\) suggests that accumulation of Ca\(^{2+}\) inside the cell might be involved in lidocaine-induced inhibition of axonal transport. Ca\(^{2+}\)-calmodulin-dependent protein kinase II (CAM II kinase), which is activated by intracellular Ca\(^{2+}\), has been implicated in regulating the organization of neuronal cytoskeleton\(^{21}\) 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 Ca\(^{2+}\) ionophore on axonal transport. The Ca\(^{2+}\) ionophore A23187 (0.1 \(\mu M\)) 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 \(\mu M\)) 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 Calmodulin-dependent Protein Kinase II Inhibitor

In the presence of the CAM II kinase inhibitor KN-62 (10 \(\mu M\)) in extracellular medium, 30 \(\mu M\) lidocaine failed to suppress axonal transport in either an 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 exam-
Anesthesiology, V 95, No 3, Sep 2001

Discussion

Using video-enhanced microscopy, we showed that lidocaine at low concentrations (0.1–1,000 μM; IC$_{50}$ = 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 current 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 Ca$^{2+}$-free extracellular medium. Therefore, extracellular Ca$^{2+}$ may be a requisite for inhibition of axonal transport. In addition, we demonstrated here that Ca$^{2+}$ ionophore A23187 inhibited axonal transport. These results suggest that the lidocaine-induced inhibition of axonal transport may result from the accumulation of Ca$^{2+}$ inside the cell. This hypothesis needs to be supported by further studies on intracellular signaling mechanisms triggered by an increase in intracellular Ca$^{2+}$ concentration. Previous biochemical studies have shown that CAM II kinase phosphorylates microtubule-associated proteins, microtubule-associated protein 2, and tau protein, leading to microtubule disassembly. $^{21}$ and that the latter causes inhibition of fast axonal transport. $^{3}$ In turn, CAM II kinase is activated by intracellular Ca$^{2+}$. Therefore, we postulated that activation of CAM II ki-

Table 1. Effects of Lidocaine on Neurite Growth

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Growth Rate (μm/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.0 ± 4.3 (112)</td>
</tr>
<tr>
<td>30 μM Lidocaine</td>
<td>1.6 ± 2.8* (93)</td>
</tr>
<tr>
<td>10 μM KN-62</td>
<td>4.3 ± 4.5 (78)</td>
</tr>
<tr>
<td>30 μM Lidocaine + 10 μM KN-62</td>
<td>4.8 ± 5.2 (63)</td>
</tr>
</tbody>
</table>

Values are mean ± SD. The number of neurites is given in parentheses. * Significantly different from control ($P < 0.05$).
LIDOCAINE INHIBITS AXONAL TRANSPORT IN DRG NEURONS

Trations induces the accumulation of intracellular Ca\(^{2+}\). Therefore, it might be possible that lidocaine at low concentrations is much higher than those used in the current study. Moreover, lidocaine has been demonstrated to inhibit Ca\(^{2+}\) current in frog DRG cells at a threshold concentration of 10 \(\mu M\). Therefore, lidocaine at low concentrations is likely to reduce the influx of Ca\(^{2+}\) through Ca\(^{2+}\) channel mechanism. One possible explanation of the inconsistency between their findings and ours is that lidocaine might affect Ca\(^{2+}\) pump or passive Ca\(^{2+}\) influx through cell membrane to modulate intracellular Ca\(^{2+}\) signaling. García-Martín and Gutiérrez-Merino\(^{38,39}\) and Garcia-Martín and al.\(^{30}\) demonstrated that lidocaine acutely inhibits Ca\(^{2+}\) pump in synaptosomal plasma membrane, and thus suggested that lidocaine can increase the Ca\(^{2+}\) concentration of neuronal cytosol. They also mentioned that the lidocaine concentration needed to produce approximately 50% inhibition of Ca\(^{2+}\) pump activity (\(K_{0.5}\)) is 0.44 \(\mu M\), but that, because of the dependence of local anesthetic–lipid membrane interaction on membrane potential, this \(K_{0.5}\) value should be lower at the cell resting membrane potential.\(^{39}\) Therefore, it might be possible that at low concentrations the Ca\(^{2+}\) channel activity is relevant to the regulation of intracellular Ca\(^{2+}\) concentration. Conditions. However, further studies are required to address this issue.

We further discuss here the relation between Ca\(^{2+}\) and axonal transport. A number of studies have shown that fast axonal transport in a variety of neuronal types is reduced during the Ca\(^{2+}\)-free extracellular condition.\(^{31-37}\) The intraneuronal injection of Ca\(^{2+}\) chelator has also shown the same effect.\(^{38}\) Curiously, the intraneuronal injection of Ca\(^{2+}\) blocks axonal transport as well.\(^{38}\) Furthermore, it has been reported that axonal transport is inhibited by Ca\(^{2+}\) ionophores\(^{39-41}\) and by chemical agents that increase concentrations of intracellular Ca\(^{2+}\).\(^{42}\) which is similar to our results. Taken together, not only reduction but also elevation in intracellular Ca\(^{2+}\) concentrations appears to be a factor to inhibit axonal transport. Thus, the elevation of intracellular Ca\(^{2+}\) concentrations could be the acceptable mechanism in mediating inhibitory action of lidocaine on axonal transport.

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 \(\mu M\)) 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 \(\mu M\)). This is supported by previous studies showing that the overexpression of CAM II kinase inhibits neurite growth.\(^{22-24}\) Our current 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 that lidocaine at low concentrations inhibited axonal transport and neurite growth. These inhibitory actions are mediated through activation of CAM II kinase.

References


Anesthesiology, V 95, No 3, Sep 2001

Downloaded From: http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/931224/ on 04/16/2017


