Direct Inhibition of Microtubule-Based Kinesin Motility by Local Anesthetics

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ABSTRACT Local anesthetics are known to inhibit neuronal fast anterograde axoplasmic transport (FAAT) in a reversible and dose-dependent manner, but the precise mechanism has not been determined. FAAT is powered by kinesin superfamily proteins, which transport membranous organelles, vesicles, or protein complexes along microtubules. We investigated the direct effect of local anesthetics on kinesin, using both in vitro motility and single-molecule motility assays. In the modified in vitro motility assay, local anesthetics immediately and reversibly stopped the kinesin-based microtubule movement in an all-or-none fashion without lowering kinesin ATPase activity. QX-314, a permanently charged derivative of lidocaine, exerted an effect similar to that of lidocaine, suggesting that the effect of anesthetics is due to the charged form of the anesthetics. In the single-molecule motility assay, the local anesthetic tetracaine inhibited the motility of individual kinesin molecules in a dose-dependent manner. The concentrations of the anesthetics that inhibited the motility of kinesin correlated well with those blocking FAAT. We conclude that the charged form of local anesthetics directly and reversibly inhibits kinesin motility in a dose-dependent manner, and it is the major cause of the inhibition of FAAT by local anesthetics.

INTRODUCTION

In addition to their anesthetic effects on excitable cell membranes, local anesthetics are known to affect a variety of cellular motile functions. They inhibit fast anterograde axoplasmic transport (FAAT) (Aasheim et al., 1974; Fink et al., 1972; Fink and Kish, 1976; Lavoie, 1982a,b, 1983; Lavoie et al., 1989), neurite outgrowth (Anderson and Bamburg, 1981), the motility of cilia (Manawadu et al., 1978), inflammatory responses such as chemotaxis or leukocyte migration (Cullen and Haschke, 1974; Sasagawa, 1991), phagocytosis (Cullen and Haschke, 1974), exocytosis (Banerjee and Redman, 1977), the endocrine system (Eichhorn and Peterkofsky, 1979; Kawabata et al., 1993), and cell proliferation (Martinsson et al., 1993). These effects are unrelated to the ability of local anesthetics to block the conduction of nerve impulses, and the precise mechanisms have not been determined (Lavoie et al., 1989).

Motility for all of these events depends on motor proteins, i.e., actomyosin, kinesin, and dynein superfamily proteins. It has been shown that local anesthetics directly inhibit actomyosin motility in vitro (Tsuda et al., 1996). Thus an inhibition of the motility of these motor proteins could be a unifying mechanism for the various inhibitory effects of local anesthetics.

FAAT is inhibited by local anesthetics in a reversible and dose-dependent manner (Fink and Kish, 1976). FAAT is powered by kinesin superfamily proteins, which transport membranous organelles, vesicles, or protein complexes along microtubules (MTs) by utilizing the chemical energy of ATP hydrolysis (Hirokawa, 1998). Therefore, local anesthetics might affect kinesin, MTs, the energy supply, or even other, unknown factors. At the concentration that leads to the inhibition of axoplasmic transport, local anesthetics neither depolymerize MTs (Byers et al., 1973, 1979; Fink et al., 1972; Lavoie et al., 1989) nor decrease the intracellular ATP or creatine phosphate as much as they inhibit FAAT (Banerjee and Redman., 1977; Lavoie et al., 1989). Therefore, the present study was designed to examine whether local anesthetics directly inhibit kinesin motility. In vitro motility assays (Vale et al., 1985) and a single-molecule imaging technique (Funatsu et al., 1995; Vale et al., 1996) have elucidated the mechanism by which local anesthetics act on individual single kinesin molecules to inhibit FAAT.

MATERIALS AND METHODS

Chemicals

Lidocaine hydrochloride (2-diethylamino-*N*-[2,6-dimethylphenyl]acetamide) and tetracaine hydrochloride (4-[butylamino]benzoic acid 2-[dimethylamino]ethyl ester) were purchased from Sigma Chemical Co. (St. Louis, MO). QX-314, a quaternary derivative of lidocaine (*N*-(2, 6-dimethylphenylcarbamoylmethyl)triethylammonium bromide), which has a permanent positive charge, was obtained from Funakoshi Co. (Tokyo, Japan), and HEPES was purchased from Dojindo (Kumamoto, Japan). All of the other chemicals were of reagent grade or higher (Wako Pure Chemicals, Osaka, Japan). All of the solutions were made with distilled deionized water.

Bovine brain kinesin and rhodamine- or Cy5-labeled MT

Bovine kinesin and tubulin were purified from fresh bovine brain by MT affinity purification, followed by DEAE chromatography and sedimenta-

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tion, as described previously (Kojima et al., 1997). Microtubule-associated protein (MAP)-free tubulin was prepared by the method of Williams et al. (Williams and Lee, 1982), with some modification (Haimo and Fenton, 1988). Tubulin was labeled with either tetramethylrhodamine (TMR) (C-1171; Molecular Probes) or Cy5 monofunctional dye (PA25001; Amersham Life Science, Arlington Heights, IL) (Hyman et al., 1991). Fluorescent MTs were prepared by copolymerization of labeled and unlabeled tubulin at a molar ratio of 1:62 and 1:72 for TMR-labeled tubulin and Cy5-labeled tubulin, respectively. For assays, MTs with a length of 5–15 μm were prepared, using a short segment of polymer as a seed for polymerization (Mitchison and Kirschner, 1984). Polymerization was performed in 80 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.8), 1 mM $MgSO_4$, 1 mM EGTA, 1 mM GTP at 35°C for 30 min, and the MT was stabilized by the addition of 0.1 mM taxol (T-7402; Sigma Chemical Co.).

Fluorescently labeled kinesin

We used human kinesin that was truncated just before the hinge, at residue 560, and a short peptide containing a reactive cysteine was introduced at its C-terminus (uhK560Cys) (Vale et al., 1996). UhK560Cys expressed in *Escherichia coli*, BL21(DE3)plysS, was purified and labeled at cysteine with Cy3-maleimide, which was obtained by linking Cy3-OSu (PA-23001; Biological Detection Systems) to *N*-(2-(1-piperazinyl)ethyl) maleimide. The stoichiometry of labeling was 1.26 dyes per polypeptide chain, as determined from the fluorescent intensity of individual kinesin spots and their photobleaching pattern in total internal reflection microscopic images (Funatsu et al., 1995; Vale et al., 1996).

Observation under conventional fluorescence microscope

For the in vitro motility assays, fluorescent MTs were imaged using a conventional inverted fluorescence microscope (TMD; Nikon. Co., Tokyo, Japan) with an oil-immersion objective lens (Neofluar, ×100, N. A. = 1.3; Carl Zeiss, Jena, Germany). The fluorescence images were projected onto a silicon-intensifier target tube (SIT) camera (Hamamatsu C-2400-08; Hamamatsu Photonics, Shizuoka, Japan) and stored in a video cassette recorder. The lengths and the sliding velocities of individual MTs were quantified with an image processor (TVIP4100; Avionics, Tokyo, Japan).

Observation under total internal reflection microscope

For the single-molecule motility assay, fluorescent single kinesin molecules were imaged using low background total internal reflection microscope (Funatsu et al., 1995, 1997; Vale et al., 1996). To visualize single kinesin molecules and fluorescent MTs simultaneously, we introduced a dual-view optics into the microscope (Kinosita et al., 1991) (Fig. 1 *a*). The

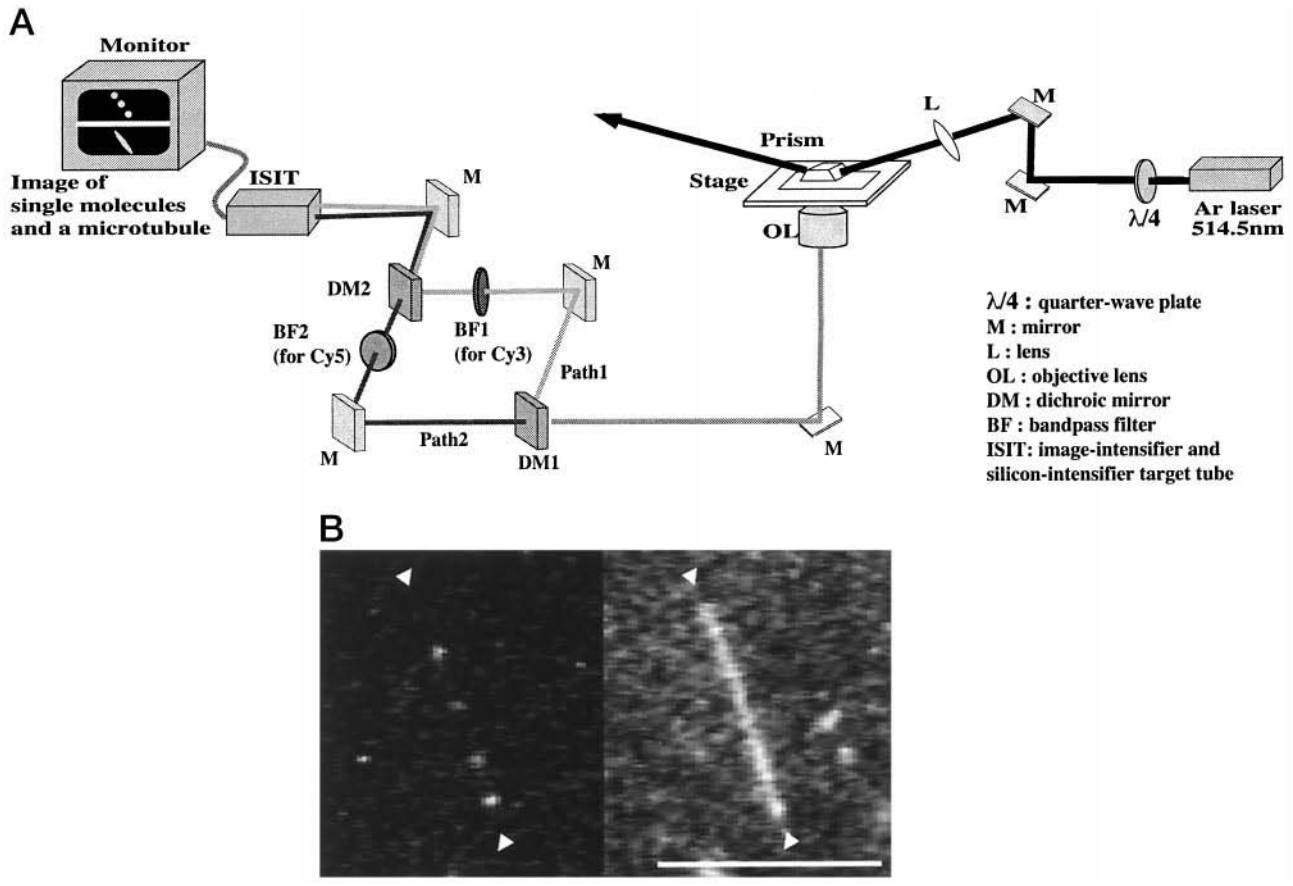


FIGURE 1 (*a*) The optical system of low background total internal reflection microscopy equipped with dual-view optics. Images of fluorescent single kinesin molecules and fluorescent MTs are projected side by side on the photocathode of the image intensifier so that they can be observed simultaneously. (*b*) Images of Cy3-labeled single kinesin molecules (Cy3-uhK560Cys) (*left*) and a Cy5-labeled MT (*right*) displayed on the monitor simultaneously. Four kinesin molecules are seen on the track of the MT (indicated between the two *arrowheads*). Scale bar, 10 μm.

fluorescence from the specimen was split by a dichroic mirror (Sigma Koki, Saitama, Japan) (DM1 in Fig. 1 *a*) into two, with a separation wavelength of 630 nm. The two beams were bandpassed filtered (570DF30 for Cy3 and 680DF55 for Cy5; Omega) to exclusively transmit the fluorescence. They were then passed through another dichroic mirror (DM2), and the two separate images were focused on the focal plane of an image intensifier (VS4-1845; Video Scope International, Sterling, VA) coupled to an SIT camera (Hamamatsu C-2400-08; Hamamatsu Photonics) and recorded with a video cassette recorder. The imaging was performed at an excitation laser power of 46 mW and an illumination area of 250 × 110 μm^2 ($1/e^2$ value). The incident angle of the beam to the norm at the interface was 68°.

Standard in vitro motility assay (kinesin molecules are densely adsorbed)

A coverslip (9 mm × 9 mm; Matsunami Co., Kishiwada, Japan) was placed on the center of another coverslip (24 mm × 32 mm; Iwaki Co.) via two strips of double-coated tape to make a flow chamber (9 mm × 5 mm × ~110 μm). Bovine brain kinesin was diluted to a density of 11 μg/ml (28.2 nM, calculated from the molecular mass of 390 kDa; Kuznetsov et al., 1988) with modified BRB80 buffer (M-BRB80; 80 mM HEPES, 2 mM $MgCl_2$, 1 mM EGTA, pH 7.4, ionic strength 0.1 M). For pH 6.4 and 6.8, piperazine-*N,N′*-bis(2-ethanesulfonic acid was used instead of HEPES. Five microliters of dilute kinesin was introduced into the flow chamber so that kinesin molecules would adhere directly to the glass surface. We did not pretreat the surface of the chamber with blocking proteins (Block et al., 1990; Howard et al., 1989) before the application of kinesin, to exclude the possibility of interaction of these blocking agents with local anesthetics. After a 3-min incubation with kinesin, the chamber was washed with 20 μl of M-BRB80. Then 50 μl of an assay solution (LA-M-BRB80, M-BRB80 containing a local anesthetic; pH and the ionic strength were adjusted to the original values) containing TMR-labeled MTs (15 μg/ml), 10 μM taxol, 1 mM ATP, and an oxygen scavenger (25 mM glucose, 36 μg/ml catalase, 220 μg/ml glucose oxidase, 1% 2-mercaptoethanol) (Harada et al., 1990) was applied to the flow chamber. In the LA-M-BRB80 buffer containing lidocaine, 80 mM HEPES (or piperazine-*N,N′*-bis(2-ethanesulfonic acid) was reduced to 20 mM to adjust the ionic strength to 0.1 M. Gliding movements of individual MTs were recorded with a video cassette recorder. The length and velocity of individual MTs were measured with a computer image processor (TVIP4100; Avionics, Tokyo, Japan).

Modified in vitro motility assay (kinesin molecules are sparsely adsorbed)

Because the movement of vesicles in vivo is powered by only a few kinesin molecules bound to their surface (Hirokawa, 1982, 1998; Miller and Lasek, 1985), we performed the above assay with a low density of kinesin at pH 7.4, to simulate in vivo conditions. The flow chamber on a coverslip was prepared as described above. Five microliters of kinesin solution diluted with M-BRB80 (400 ng/ml = 1.1 nM) was introduced into the flow chamber. After a 3-min incubation with kinesin, the chamber was washed with 20 μl of M-BRB80. Then 50 μl of M-BRB80 containing TMR-labeled MTs (15 μg/ml), 10 μM taxol, 1 mM ATP, and the oxygen scavenger was applied. After it was confirmed that more than 50% of the MTs were gliding, the chamber was washed with 50 μl of LA-M-BRB80 containing the same concentrations of TMR-labeled MTs, taxol, ATP, and the oxygen scavenger. Finally, to test for reversibility, the chamber was washed again with 50 μl of M-BRB80 containing the same concentrations of TMR-labeled MTs, taxol, ATP, and the oxygen scavenger. The images of the MTs were recorded, and the length and the velocity of individual MTs were measured by the computer image processor as described above. Local anesthetic treatment did not lead to MT depolymerization throughout our study, because of the MT-stabilizing property of taxol.

Single-molecule motility assay

Quartz glass slides (26 mm × 56 mm; Matsunami Co.) were cleaned with 0.2 M KOH, 100% acetone, and air dried. They were subsequently treated with silicone solution (SL-2, Sigma Co., 5% (v/v) in heptane). Twenty microliters of Cy5-labeled MT (10 μg/ml) in buffer A (20 mM HEPES, 10 mM potassium acetate, 4 mM $MgSO_4$, 2 mM EGTA, 0.2 mM EDTA; pH 7.4; ionic strength 29 mM) containing 10 μM taxol was placed on the silicone-treated quartz glass slide between two strips of thin polyester film (25 μm thickness, 22 mm × 2 mm), and immediately covered with a coverslip (18 mm × 18 mm, Matsunami Co.). Thus, a flow chamber was produced between the quartz glass and the coverslip. After a 5-min incubation to allow the MTs to attach to the surface of the quartz glass, the chamber was washed with 30 μl of casein solution (5 mg/ml) and incubated for another 5 min, to block the nonspecific binding of kinesin molecules to the surface of the quartz glass. The chamber was subsequently washed with 50 μl of buffer A containing 1 mM ATP and 10 μM taxol. Finally, 30 μl of kinesin (2 nM uhK560-Cy3) in buffer A containing tetracaine hydrochloride (pH 7.4, ionic strength 29 mM), 1 mM ATP, 10 μM taxol, and the oxygen scavenger was added to the flow chamber. The polyester sheets were removed, and the flow chamber was sealed with nail enamel. Under the total internal reflection fluorescence microscope with dual-view optics, fluorescent images of single kinesin molecules and MTs were observed and recorded simultaneously at a full video rate (Fig. 1 *b*).

The location and the MT-based movements of individual kinesin molecules were quantified from the tape recordings, using a computer image processor system (Swallow; Inter Quest, Osaka, Japan). Only the movements on long MTs (9–12 μm) were measured to minimize the chance that a Cy3-kinesin would release from a MT simply by running off its end. At the concentration of kinesin (~2 nM), kinesin binding on an individual MT did not overlap, so that single fluorescent spots could easily be distinguished. The number of the association events per minute per micron of MT was 3.8 ± 0.5, 2.7 ± 0.6, and 3.1 ± 1.1 (mean ± SD) for 0, 2.5, and 5 mM tetracaine, respectively, whereas only 0.03 ± 0.01 events per min were scored on glass without a MT.

The duration of the shot noise of the SIT camera was examined, and its distribution was fit with a Gaussian curve (0.12 ± 0.06 s, mean ± SD). To minimize the chance of mistaking shot noise for a fluorescent kinesin molecule, only the fluorescent spots that stayed on the track of the MT for more than 0.24 s (mean + 2 SD of the shot noise) were regarded as kinesin molecules.

Photobleaching and blinking of fluorescent molecules (Dickson et al., 1997) and the effect of local anesthetic tetracaine on these processes were also examined. For these measurements, Cy3-kinesin molecules were adsorbed directly to a quartz glass slide, and the time required for a single fluorescent spot to disappear for the first time by either photobleaching or blinking was examined. The distribution of the disappearance of the fluorescent spots should decay exponentially (Vale et al., 1996). At the laser power used in this assay (46 mW), the rate of disappearance (k_{dis}) in the absence and the presence of 5 mM tetracaine was 0.084 ± 0.001 s^{-1} and 0.080 ± 0.001 s^{-1}, respectively. In the single-molecule motility assay, kinesin spots moving along a MT disappeared at 0.77 ± 0.02 s^{-1} and 1.64 ± 0.04 s^{-1} in the absence and the presence of 5 mM tetracaine, respectively (k_{obs}; observed rate of disappearance). They were significantly larger than the k_{dis} values for kinesin attached to glass. Thus no correction was required for photobleaching and blinking. From the k_{obs} and k_{dis}, the actual run length of the motor in the absence of Cy3 photobleaching and blinking, k_{rel} (motor release constant), could be calculated from the equation $k_{obs} = k_{rel} + k_{dis}$ (Romberg et al., 1998). We did not correct the data using this equation because k_{dis} values were small. All of the experiments, including the in vitro motility assay, were performed at 25°C.

ATPase measurements

Microtubule-activated ATPase activity of kinesin (uhK560) was measured at 25°C in buffer A containing tetracaine (pH 7.4, ionic strength 29 mM) or in M-BRB80 containing lidocaine or QX-314 (pH 7.4, ionic strength

100 mM). Ten micromolar taxol and 1 mM dithiothreitol (DTT) were added to the buffers. The reaction was initiated by the addition of 2 mM ATP and terminated with the addition of perchloric acid. Inorganic P_i was detected by the modified malachite green method (Ohno and Kodama, 1991). MT-activated ATPase activity of unlabeled kinesin (uhK560) has been demonstrated to be similar to that of Cy3-labeled kinesin (uhK560-Cy3) (Vale et al., 1996). Neither chemical reactions of the assay nor the absorbance of malachite green at 650 nm was affected by the anesthetic. The values of k_{cat} and $K_{0.5,MT}$ were determined from the hyperbolic fit.

RESULTS

Dose-dependent decrease in MT gliding velocity by local anesthetics in the standard in vitro motility assay

In the standard in vitro motility assay, the gliding velocity of MTs decreased as the concentration of the anesthetics increased (Figs. 2, *a* and *b*), and the effect was totally reversible. The measure of the inhibitory potency of the anesthetics, the decrease in MT gliding velocity (% per mM anesthetic), at pH 7.4 was only 1.5 times greater than that at pH 6.4 in the case of tetracaine and only 1.2 times greater in the case of lidocaine (see Discussion). All of the MTs on the surface moved smoothly and continuously, even in the presence of the anesthetics. These effects are not large enough to explain the inhibition of FAAT by local anesthetics.

Local anesthetics stop the gliding of MTs in the modified in vitro motility assay (kinesin molecules are sparsely adsorbed)

A single kinesin molecule can move a MT for several micrometers as quickly as several kinesin molecules can (Howard, 1996). In the standard in vitro assay described above, gliding movement of a MT is supported by many kinesin molecules. The number of kinesin molecules, the motility of which is affected by the anesthetics, would fluctuate around the equilibrium. Therefore, it is possible that even if the motility of the majority of kinesin molecules is affected, a small number of remaining intact molecules could still move the MT; i.e., an inhibitory effect of local anesthetics on a subset of kinesin could be dulled by the remaining kinesin molecules with intact motility. We examined this possibility by observing the effects of local anesthetics on MT gliding movements, which are supported by only a few kinesin molecules.

In this experiment, kinesin molecules were sparsely adsorbed to the coverslip as described. Because of the low density of kinesin, only a few kinesin molecules would be expected to interact with a MT; thus not all of the MTs glide, even in the absence of an anesthetic. When a buffer containing a local anesthetic (5 mM tetracaine hydrochloride, 50 mM lidocaine hydrochloride, or 50 mM QX-314) was applied to the sample, almost all of the MTs immediately stopped gliding (Fig. 3, *a-c*). When the anesthetic was removed by applying the control buffer, most MTs recovered their motility instantaneously. The velocity of gliding MTs after the removal of the anesthetic did not differ from that before the application of the anesthetic, indicating the full reversibility of the effect (Table 1). The length of the MTs examined for the analysis did not differ among the three states (before the application of an anesthetic, in the presence of the anesthetic, and after the removal of the anesthetic). The effect of QX-314, a permanently charged derivative of lidocaine, was similar to that of lidocaine (Fig. 3 *c*).

At the concentrations lower than those described above (1, 2, 3, and 4 mM for tetracaine; 10, 20, 30, and 40 mM for

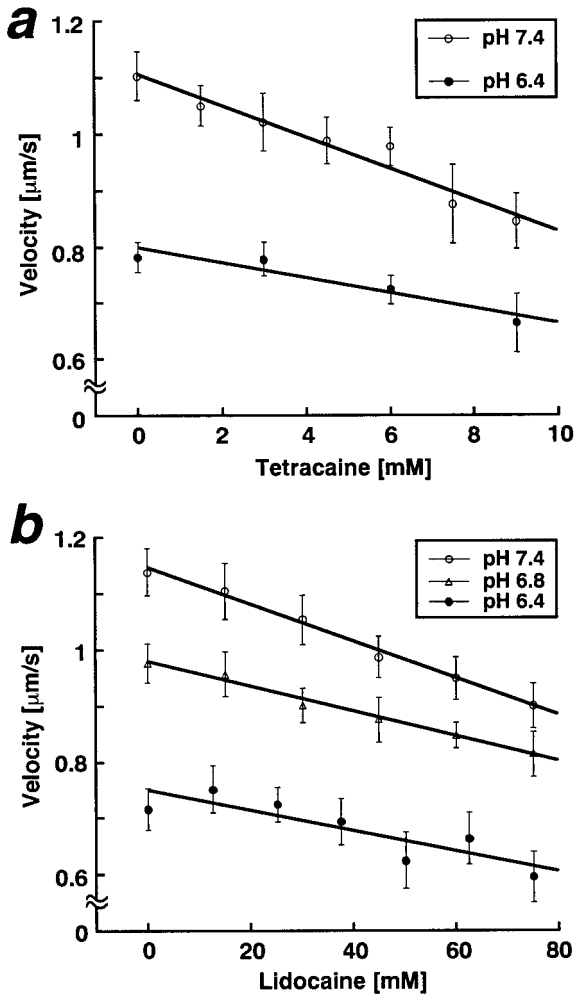


FIGURE 2 The gliding velocity of MTs in the standard in vitro motility assay, in the presence of (*a*) tetracaine and (*b*) lidocaine at various levels of pH. Bovine brain kinesin was used in this assay. In the case of tetracaine, the decrease in MT gliding velocity (% per mM anesthetic) was 2.5 and 1.7 for pH 7.4 and pH 6.4, respectively. In the case of lidocaine, it was 0.29, 0.23, and 0.24 for pH 7.4, pH 6.8, and pH 6.4, respectively. Gliding velocity was measured only for the MTs that moved continuously for more than15 s and averaged for 50–100 different MTs in each experiment. The entire experiment was performed at 25°C. Declined velocities were recovered to the original levels by the removal of the anesthetics. Bars, SD.

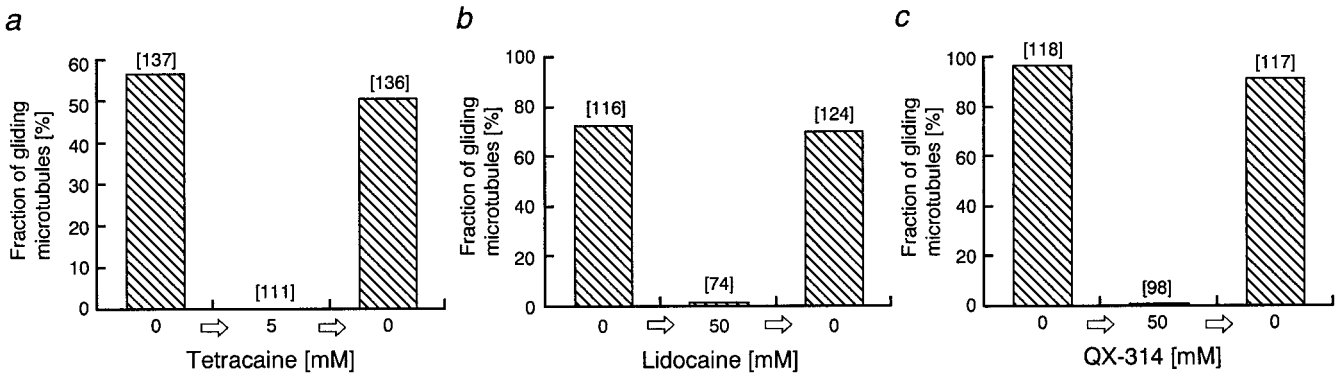


FIGURE 3 The proportion of gliding MTs in the modified in vitro motility assay, in the presence of (*a*) tetracaine, (*b*) lidocaine, and (*c*) QX-314. Bovine brain kinesin was used in this assay. The initial percentage of gliding MTs depends on the concentration of kinesin that adhered onto the coverslip. The actual number of MTs examined is provided above the respective data.

lidocaine and QX-314), local anesthetics exerted no significant effects on MT gliding. Thus the effect was in an all-or-none fashion with a certain threshold (50 mM for lidocaine and QX-314, 5 mM for tetracaine).

Local anesthetic tetracaine suppresses the movement of individual single kinesin molecules in a dose-dependent manner

Although the local anesthetics inhibited the kinesin-based MT gliding in an all-or-none fashion in the above experiment, these results do not necessarily imply that they inhibit the motility of individual kinesin molecules in an all-or-none fashion. An all-or-none inhibition of MT gliding movement could have resulted from the nature of the experimental system (see Discussion).

Therefore, the effect of the local anesthetic tetracaine on the movements of single kinesin molecules was directly examined by using the single molecule motility assay. For individual kinesin molecules, the duration of interaction with a MT and the travel distance before release from the MT were measured (Fig. 4, *a–c*). The distributions of the travel distance were fitted with single-exponential decays (Fig. 4, *a–c*, *insets*), which gave the mean travel distances (decay constant B in the figure legend). Tetracaine decreased the travel distance in a dose-dependent manner (Fig. 4 *d*), and the concentration of tetracaine that decreases the mean travel distance to $1/e$ (K_I) was 5.1 mM. In the presence of 5 mM tetracaine, most of the molecules bound to the MT showed little motility, matching the result of the modified in vitro motility assay, in which 5 mM tetracaine was exactly the threshold at which microtubule gliding stopped (Fig. 3 *a*). The K_I values of lidocaine and QX-314 would be also similar to the thresholds at which microtubule gliding stopped, ~50 mM.

TABLE 1 Velocity of gliding MTs and the length of examined MTs

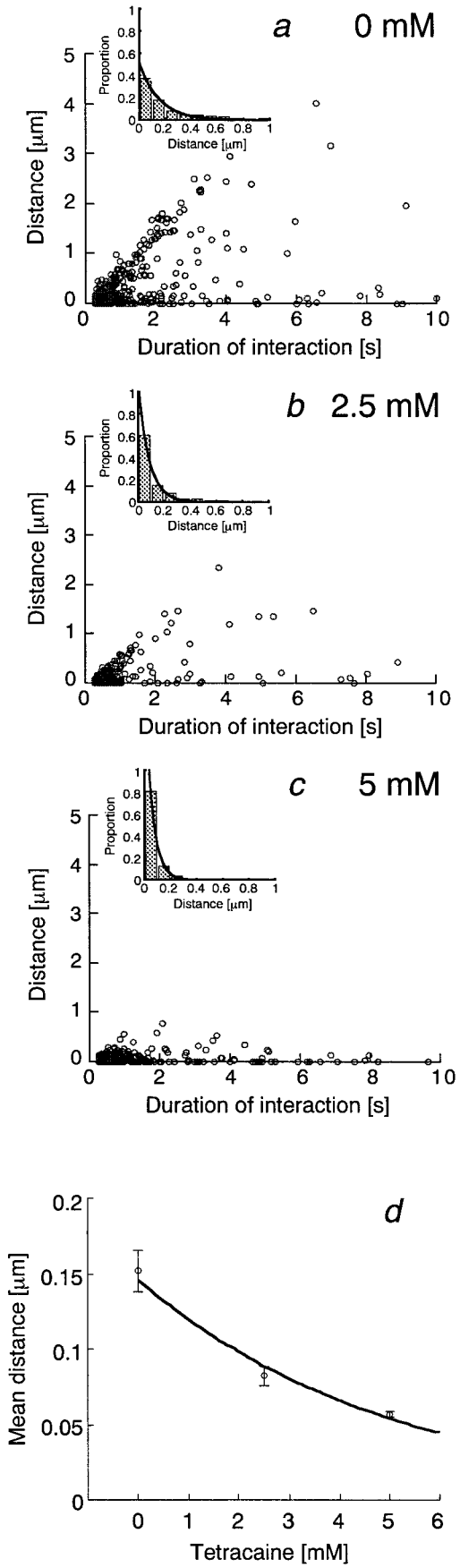
	Velocity of gliding MTs (μm/s)	Length of MTs (μm)
Tetracaine		
0 mM	0.84 ± 0.25*	5.3 ± 2.8‡
0 ➡ 5 mM	—	5.9 ± 2.9‡
5 ➡ 0 mM	0.89 ± 0.23*	5.5 ± 3.0‡
Lidocaine		
0 mM	0.89 ± 0.23†	5.7 ± 3.0§
0 ➡ 50 mM	—	6.2 ± 2.9§
50 ➡ 0 mM	0.85 ± 0.17†	6.0 ± 2.9§
QX-314		
0 mM	0.95 ± 0.19¶	6.1 ± 2.9‖
0 ➡ 50 mM	—	6.0 ± 2.8‖
50 ➡ 0 mM	0.90 ± 0.10¶	6.9 ± 2.7‖

Note: Values are mean ± SD.
*, †, ‡, §, $p > 0.1$ (Student's *t*-test).
¶, ‖, $p > 0.01$ (Student's *t*-test).

The rate of binding of uhK560 to the MT was not significantly influenced by tetracaine. The number of the association events per minute per micrometer of MT was 3.8 ± 0.5, 2.7 ± 0.6, and 3.1 ± 1.1 (mean ± SD) for 0, 2.5, and 5 mM tetracaine, respectively (10-min observation for each MT). The rate of dissociation of kinesin from the MT (the reciprocal of the duration of interaction with a MT) was doubled by the presence of 5 mM tetracaine (0.77 ± 0.02 s^{-1} and 1.64 ± 0.04 s^{-1} in the absence and in the presence of 5 mM tetracaine, respectively).

Local anesthetics do not interfere with the ATPase activity of kinesin

Because kinesin moves along a MT by using the free energy liberated by the hydrolysis of ATP, the effect of local anesthetics on the MT-activated kinesin ATPase activity was investigated. Neither k_{cat} nor $K_{0.5,MT}$ of uhK560 was affected by the presence of the anesthetics at the concentrations that promote maximum inhibition of kinesin motility (Fig. 5).



DISCUSSION

Local anesthetics prevent kinesin from moving forward

There are three possible mechanisms by which kinesin-based MT gliding could be affected by local anesthetics. Local anesthetics might 1) dissociate kinesin-MT complex, 2) lower the velocity of individual kinesin molecules, or 3) stop the movement of kinesin without dissociating it from the MT.

In the standard in vitro motility assay with a relatively high density of kinesin on the coverslip, the gliding velocity of MTs was decreased by local anesthetics in a dose-dependent manner (Fig. 2). If the anesthetics were only to dissociate the kinesin-MT complex, the number of intact kinesin molecules interacting with a MT would have decreased, but the MT gliding velocity would not have decreased, because the remaining unaffected kinesin molecules could move the MT at normal velocity (Howard et al., 1989). Thus our result implies that anesthetics either lower the velocity of individual kinesin molecules in a dose-dependent manner or prevent kinesin from moving forward without dissociating it from the MT, imposing a little drag on the MT gliding movement. To test these two possibilities, we had to lower the number of kinesin molecules participating in the movement of a MT. Another reason to lower the number of kinesin molecules is that the standard motility assay does not mimic the in vivo situation well. The movement of vesicles in vivo is powered by only a few kinesin molecules bound to their surface (Hirokawa, 1982, 1998; Miller and Lasek, 1985), whereas in the standard motility assay, a large number of kinesin molecules are involved in the movement of a single MT.

In the modified in vitro motility assay with low density of kinesin on the coverslip, local anesthetics stopped the gliding movement of MTs in an all-or-none fashion. If the

FIGURE 4 (*a–c*) The travel distance of individual kinesin molecules (Cy3-uhK560Cys) on a MT before being released from it and their duration of interaction with the MT in the presence of (*a*) 0 mM, (*b*) 2.5 mM, and (*c*) 5 mM tetracaine. Each open circle represents the data for a single kinesin molecule. *Inset*: The distribution of travel distance of individual kinesin molecules. The vertical axis indicates the proportion to the total number of the molecules investigated. The distributions were fitted with exponential decays:

$$\text{Proportion} = Ae^{-(\text{Distance}[\mu\text{m}])/B}$$

where B indicates the decay constant for the travel distance. Tetracaine decreased the constant B in a dose-dependent manner: 0.152 ± 0.014 μm, 0.082 ± 0.006 μm, and 0.057 ± 0.002 μm for 0 mM, 2.5 mM, and 5 mM tetracaine, respectively. In the presented experiments, 378, 245, and 376 molecules were retained for analysis for 0 mM, 2.5 mM, and 5 mM tetracaine, respectively (see text). (*d*) The mean travel distance of individual single kinesin molecules (the decay constant B) plotted as a function of tetracaine concentration. It was fitted with a single exponential curve, and the decay constant was 5.1 mM. Bars, SD.

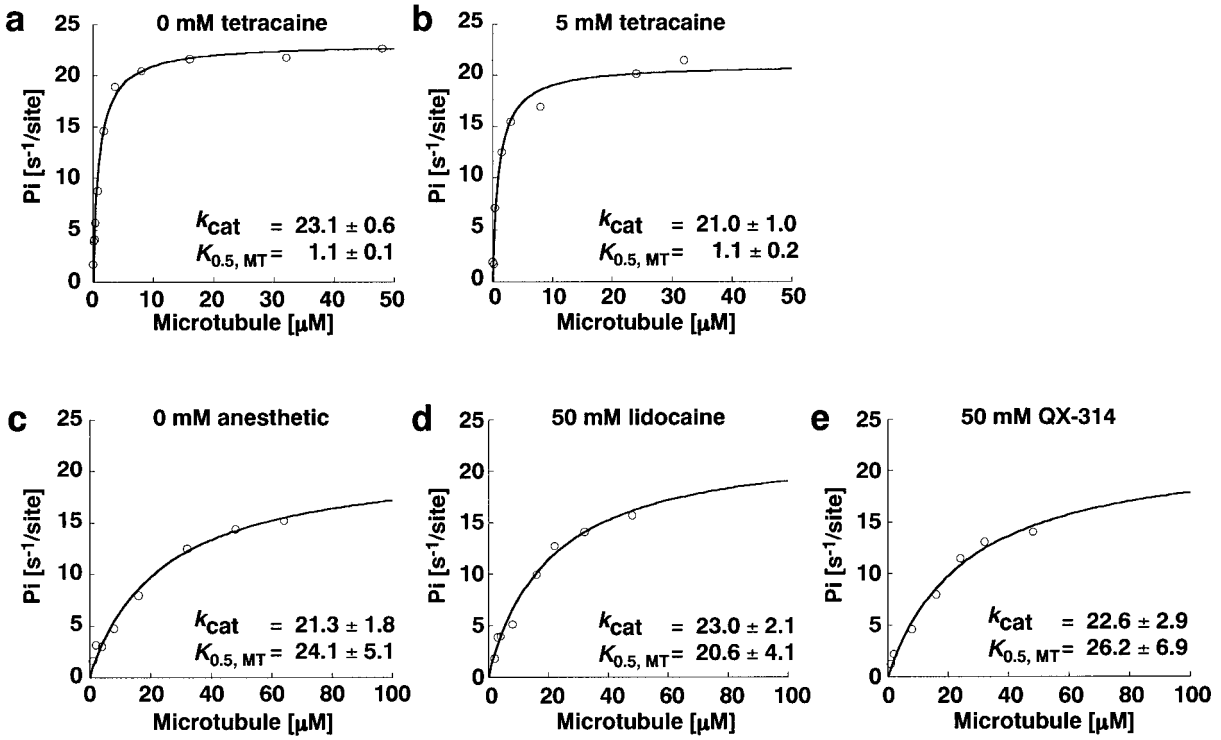


FIGURE 5 Microtubule-activated ATPase activity of kinesin (uhK560Cys) measured at 25°C in buffer A (*a–b*, pH 7.4, ionic strength 29 mM) or in M-BRB80 (*c–e*, pH 7.4, ionic strength 100 mM). The values of k_{cat} and $K_{0.5,MT}$ were determined from the hyperbolic fit. The difference in $K_{0.5,MT}$ between *a–b* and *c–e* is due to the difference in the ionic strength.

anesthetics only dissociated the kinesin-MT complex, MTs would have been dissociated from the kinesin-coated surface, instead of being stopped. If they lowered the velocity of individual kinesin molecules in a dose-dependent manner, the gliding velocity of MTs would have been gradually lowered, instead of being abruptly stopped. Thus these data suggest that local anesthetics prevent kinesin from moving forward without completely dissociating it from the MT.

In the single-molecule motility assay, we observed that the distance that a kinesin could move along a MT decreased as the concentration of tetracaine increased, and almost all of the motion was abolished at 5 mM tetracaine, matching the result we obtained with the modified in vitro motility assay. Although the dissociation rate of individual kinesin molecules from a MT (the reciprocal of the duration of interaction with the MT) was doubled by 5 mM tetracaine, the dissociation rate of the MT from the kinesin-coated glass would not be doubled, because under this condition there is more than one kinesin molecule attached to the MT.

Charged form of local anesthetics is responsible

The ratio of the charged form to the uncharged form of a tertiary amine local anesthetic is given by the Henderson-Hasselbalch equation,

$$pK_a = pH + \log \frac{[BH^+]}{[B]}$$

where $[BH^+]$ and [B] are the concentrations of the charged and the uncharged forms, respectively. The pK_a values for tetracaine and lidocaine are, respectively, 8.59 and 8.19 at 25°C (Kamaya et al., 1983). Thus 93.9% of tetracaine and 86.1% of lidocaine are charged at pH 7.4, and as the pH increases from 6.4 to 7.4, the uncharged form of the anesthetics increases by ninefold. However, in the standard in vitro motility assay, the inhibition of MT gliding by increases in the concentration of the anesthetics changed only 1.5 times at maximum with this change in pH, implying that the inhibitory effect was not due to the uncharged form of the anesthetics.

In the modified in vitro motility assay, the inhibition of motility by QX-314 (Fig. 3 *c*) suggested that the charged form of local anesthetics is responsible for inhibiting kinesin motility. This contrasts with the result obtained with actomyosin, despite the structural similarity to kinesin (Howard, 1997; Kull et al., 1996); myosin-based actin motility is interfered with by the uncharged form of local anesthetics (Tsuda et al., 1996).

When a more uncharged form is present, local anesthetics have been reported to be more potent in blocking FAAT (Lavoie, 1982b), contrasting with our result. When an anesthetic is applied to the neuronal cell, the charged form cannot go through the membrane, but the uncharged form penetrates the plasma membrane; thus a new equilibrium between the charged and uncharged forms must be established inside the cell. Therefore, the uncharged form would be expected to be more potent when applied to the outside of the cell. However, inside the cell, our results demonstrate that the charged form of the anesthetic inhibits kinesin motility.

Local anesthetics uncouple kinesin's mechanical event from its biochemical energy source

From the raw data of Fig. 4 *a* (0 mM tetracaine), assuming that a kinesin molecule hydrolyzes one ATP per 8-nm step (Hua et al., 1997; Schnitzer and Block, 1997) and stationary kinesin bound to a MT does not hydrolyze ATP, the calculated rate of ATP hydrolysis of kinesin molecules interacting with the MT is 27.1 $\pm$ 0.1 s^{-1}/site, which is consistent with the k_{cat} value of our ATPase measurement (23.1 $\pm$ 0.6 s^{-1}/site). In contrast, in the presence of 5 mM tetracaine (from the raw data of Fig. 4 *c*), the calculated rate of ATP hydrolysis is 6.69 $\pm$ 0.03 s^{-1}/site, which is much lower than our measured k_{cat} (21.1 $\pm$ 1.0 s^{-1}/site). Thus these results suggest that local anesthetics uncouple kinesin's mechanical event from its biochemical energy source, and as the concentration of the anesthetic is increased, the number of kinesin molecules that hydrolyze ATP without moving forward increases.

Which process of kinesin-MT interaction do local anesthetics affect?

The charged form of local anesthetics inhibits neither the binding of kinesin to MTs nor ATP hydrolysis, but rather uncouples ATP hydrolysis from movement and inhibits kinesin's ability to move along a MT. Local anesthetics do not bind to purified tubulin in vitro (Eichhorn and Peterkofsky, 1979), and thus it could be possible that the hydrophilic structure of kinesin or the hydrophilic interaction between kinesin and MT is disrupted by the charged form of local anesthetics.

The mechanism for dimeric kinesin movement along a MT in a processive manner is controversial (Block, 1998). The prevailing model for processivity is the so-called hand-over-hand model, in which the motor remains in continuous contact with the MT as a result of an alternating interaction of the two heads (Hackney, 1994; Hirose et al., 1996; Ma and Taylor, 1997). Although it is not clear whether a hand-over-hand mechanism is essential for processivity (Okada and Hirokawa, 1999), at least for the normal motility of two-headed kinesin, coordinated motion of the two heads is considered to be important (Jiang et al., 1997; Kozielski et al., 1997; Romberg et al., 1998).

Several recent models have proposed a mechanochemical cycle for two-headed kinesin in which a sophisticated communication between its two heads is mediated by the "neck" region that connects them (Hackney, 1994; Hirose et al., 1996; Tripet et al., 1997). Three-fifths of the neck region is believed to unwind during motility to allow the MT-free head to rotate on its axis (neck) and attach to the next binding site of the MT after ATP hydrolysis (phosphate release) (Crevel et al., 1996; Hirose et al., 1995).

A notable feature of the neck region is the placement of destabilizing residues with low hydrophobicity at the interface of the two heads; 16 of the 33 residues of the region (residues 338–370 in the human kinesin uhK560) are charged, and they are highly conserved among species (Navone et al., 1992). We speculate that the charged form of local anesthetics might interfere with the interaction among the charged residues of the neck region and inhibit unwinding, resulting in an inability of the free head to rotate and step forward. Local anesthetics could inhibit the rotation of the kinesin molecule, the MT-attached head of which is in an ADP-bound "weak binding" state. Such a mechanism for the inhibition of motility is consistent with our results as follows. 1) If the head cannot move along a MT but undergoes association-dissociation and hydrolyzes ATP for each dissociation at a single position on the MT, the ATPase activity would not be affected. 2) The "weak binding" of a single kinesin molecule to a MT could impose a slight drag on movement. In the modified in vitro motility assay, there are only a few kinesin molecules attached to a MT. Thus when a local anesthetic, the concentration of which exceeds a certain threshold, is applied, all of the kinesin molecules that are attached to a single MT will be affected simultaneously, and the gliding movement of the MT suddenly stops (Fig. 3). Under the threshold, a decrease in the gliding velocity is hardly observed, because only a few molecules that are affected by the anesthetic can impose only a little drag on MT gliding movement. Therefore the effect of an anesthetic appeared in an all-or-none fashion. On the contrary, in the standard assay in which kinesin is densely adsorbed, a large number of kinesin molecules that are attached to a single MT are affected by local anesthetics. They impose a larger drag on MT gliding movement, which is proportional to the number of affected kinesin molecules; thus a dose-dependent decrease in the MT gliding velocity is observed (Fig. 2).

Single-molecule motility assay, beads movement assay, and FAAT

Organelle and vesicle transport in vivo, FAAT, is so efficient that individual particles can move at their maximum rate of transport almost continuously for many hours and

over distances of many centimeters (Brady, 1984). Reduction in the number of intact kinesin molecules per particle surface leads either to its failure to bind to a MT or to nonsuccessive movement on a MT (Block et al., 1990), which means a decrease in the amount of FAAT.

In our single-molecule motility assay, kinesin molecules that could not move on a MT increased as the concentration of tetracaine increased. This result is equivalent to the decrease in the number of intact kinesin molecules per particle surface, which leads to the inhibition of FAAT. Furthermore, tetracaine doubled the dissociation rate of individual kinesin molecules from the MT (the reciprocal of the duration of interaction with a MT), which would also lead to an inhibition of FAAT. Therefore, our results imply that the inhibition of FAAT by local anesthetics is due to an inhibition of kinesin motility.

Comparison with the whole cell study

The concentrations of the anesthetics that inhibited kinesin motility correlate well with those previously reported to block FAAT, although a precise comparison is difficult because of differences in experimental conditions.

In our experiments, 5 mM tetracaine or 50 mM lidocaine (25°C, pH 7.4, ionic strength 100 mM) inhibited the motility of kinesin molecules almost completely. Whereas in whole cell studies, 1 mM tetracaine (25°C, pH 7.1) produced ~80% inhibition of the transport (Lavoie, 1982b), and 22.2 mM lidocaine (38.5°C, pH 7.48) produced approximately 90% inhibition of transport(Fink et al., 1972). These investigators (Fink and Kish, 1976) have also reported that 20 to 30 mM lidocaine had almost completely abolished the FAAT in the trigeminal nerve of rats in vivo. Thus, there is a good agreement between the results from whole cell and individual molecules, and these trivial differences may be due to the difference in experimental conditions (pH, temperature, and ionic strength).

Another drug that inhibits kinesin motility

A natural product of the marine sponge *Haliclona*, adociasulfate-2, inhibits kinesin motility with a mechanism different from that of local anesthetics. It targets the kinesin motor domain by mimicking MT activity and abolishes kinesin's binding to a MT in a competitive manner (Sakowicz et al., 1998). Therefore, in this case, the MT-stimulated kinesin ATPase is also inhibited. Two types of kinesin inhibitors, local anesthetics and adociasulfate-2, are both useful in various experiments. An advantage of local anesthetics over adociasulfate-2 is that they can permeate intact cell membranes, whereas adociasulfate-2 is membrane impermeant because of its large size and two charged sulfate moieties.

CONCLUSION

We conclude that local anesthetics directly and reversibly inhibit the MT-based kinesin motility without lowering its ATPase activity. This action is due to the charged form of local anesthetics. The charged form of the anesthetic prevents MT-attached kinesin from moving forward, probably by interfering with the kinesin neck region. The model we present here imitates the situation in a nerve cell, so that the direct inhibition of the kinesin motility is the major cause of the inhibition of FAAT by local anesthetics.

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