Direct Inhibition of the Actomyosin Motility by Local Anesthetics in Vitro

Yuri Tsuda,*# Takashi Mashimo,* Ikuto Yoshiya,* Kuniyoshi Kaseda,§ Yoshie Harada,# and Toshio Yanagida#¶
*Department of Anesthesiology, Osaka University Medical School, Suita, Osaka 565, Japan, #Yanagida Biomotron Project, Exploratory Research for Advanced Technology, Reseach Development Corporation of Japan, Senbahigashi 2-4-14, Mino, Osaka 562, Japan, §Laboratory of Molecular Enzymology, Kyushu Institute of Technology, Ihizuka, Fukuoka 820, Japan, and ¶Department of Biophysical Engineering, Osaka University, Toyonaka, Osaka 560, Japan

ABSTRACT Using a recently developed in vitro motility assay, we have demonstrated that local anesthetics directly inhibit myosin-based movement of single actin filaments in a reversible dose-dependent manner. This is the first reported account of the actions of local anesthetics on purified proteins at the molecular level. In this study, two tertiary amine local anesthetics, lidocaine and tetracaine, were used. The inhibitory action of the local anesthetics on actomyosin sliding movement was pH dependent; the anesthetics were more potent at higher pH values, and this reaction was accompanied by an increased proportion of the uncharged form of the anesthetics. QX-314, a permanently charged derivative of lidocaine, had no effect on actomyosin sliding movement. These results indicate that the uncharged form of local anesthetics is predominantly responsible for the inhibition of actomyosin sliding movement. The local anesthetics inhibited sliding movement but hardly interfered with the binding of actin filaments to myosin on the surface or with actomyosin ATPase activity at low ionic strength. To characterize the actomyosin interaction in the presence of anesthetics, we measured the binding and breaking force of the actomyosin complex. The binding of actin filaments to myosin on the surface was not affected by lidocaine at low ionic strength. The breaking force, measured using optical tweezers, was ~1.5 pN per μm of an actin filament, which was much smaller than in rigor and isometric force. The binding and breaking force greatly decreased with increasing ionic strength, indicating that the remaining interaction is ionic in nature. The result suggests that the binding and ATPase of actomyosin are governed predominantly by ionic interaction, which is hardly affected by anesthetics; whereas the force generation requires hydrophobic interaction, which plays a major part of the strong binding and is blocked by anesthetics, in addition to the ionic interaction.

INTRODUCTION

The precise mechanisms of the effects of local anesthetics on a variety of cellular physiological functions remain unknown. Despite the widespread clinical use of local anesthesia since the phenomenon was first described by Koller and Freud in 1884 (Vandam, 1987), the electrophysiological basis for the action of local anesthetics on nerves was established only within the past several decades. The majority of investigations on the effects of anesthetics have focused on Na^+ channels (Narahashi et al., 1970; Schwarz et al., 1977; Hille, 1977; Schneider and Dubois, 1986; Chernoff, 1990; Wasserstrom et al., 1993; Zamponi and French, 1993; Wang et al., 1994; Ragsdale et al., 1994), and others deal with the full range of activities on neural functions including those of the central nervous system (Elliott and Haydon, 1989; Chernoff and Strichartz, 1989; Feldman et al., 1991; Rosenberg et al., 1993), cardiac tissue (Courtney, 1986; Moller and Covino, 1992; Reiz and Nath, 1986), and immunoreactive cells (Takagi et al., 1983; Sasagawa, 1991; Cederholm, 1994). To date no study has characterized the actions of local anesthetics upon purified proteins at the molecular level. In this study, we used recently developed biophysical techniques to characterize the direct interaction of local anesthetics on protein molecules and the resultant modifications of the protein-protein interactions at the molecular interface.

Rapid progress has been made in the biophysical investigations of the chemomechanical enzyme, actomyosin. In vitro motility assays, which address the elementary process of chemomechanical energy transduction directly at the molecular level, have contributed substantially to this progress (Huxley, 1990). In vitro motility assays have made it possible to study the actomyosin molecular motor in a simple and highly controlled way using only purified proteins. Single actin filaments labeled with fluorescent phalloidin (an actin-specific heptapeptide mycotoxin), which stabilizes the filament structure without affecting its functions, can be clearly and continuously seen by fluorescence microscopy (Yanagida et al., 1984). This technique has enabled direct observation of the movement of single actin filaments on myosin or its subfragments bound to an artificial surface (Kron and Spudich, 1986; Toyoshima et al., 1987; Harada et al., 1987).

Using this assay, we examined the modifications of the motility of actin filaments caused by two tertiary amine local anesthetics, lidocaine and tetracaine. We believe this is the first demonstration that local anesthetics directly affect the functions of purified proteins, which may give greater

Received for publication 3 January 1996 and in final form 30 July 1996.
Address reprint requests to Dr. Yuri Tsuda, Biomotron Project, ERATO, JRDC, 2-4-14, Senbahigashi, Mino, Osaka, 562, Japan Tel.: 81-727-28-7003; Fax: 81-727-28-7033; e-mail:tsuda@yanagida.jrdc.go.jp.

0006-3495/96/11/2733/09 $2.00

insights into the mechanisms of action of local anesthetics on a whole body system.

MATERIALS AND METHODS

Sample preparations

Myosin was extracted from rabbit skeletal muscle using Guba-Straub solution and purified by repeating cycles of precipitation at low ionic strength and dissolution at high ionic strength, as previously described (Yanagida and Oosawa, 1978). Two types of subfragments of myosin, heavy meromyosin (HMM) and subfragment 1 (S1), were prepared by α-chymotryptic digestion as described by Weeds and Taylor (1982) and papain digestion as described by Margossian et al. (1982), respectively. Actin filaments (2.5 μM in actin monomers) were labeled with phalloidin-tetramethyl-rhodamine (PHDTMR) by overnight incubation at 4°C in a solution containing 5 μM PHDTMR, 100 mM KCl, 10 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.0 (Yanagida, 1984). Before use in each experiment, the filaments were diluted to a final actin monomer concentration of 24 nM.

ATPase measurements

Actin-activated ATPase activities of myosin subfragments, HMM and S1, were measured in a standard assay buffer (25 mM KCl, 5 mM $MgCl_2$ and 20 mM HEPES, pH 7.8) at 30°C. The reaction was initiated by the addition of ATP and terminated by the addition of perchloric acid. Inorganic Pi was detected by the modified Malachite-Green method (Ohono and Kodama, 1990). Neither chemical reactions of Pi assay nor the absorbance of Malachite-Green at 650 nm was affected by local anesthetics. The values of V_{max} and K_m were determined by the Lineweaver-Bark plots.

Binding of S1 to F-actin

F-actin of 10 μM in monomer concentration and S1 of 5 μM were mixed in a buffer containing 0–100 mM KCl, 2 mM ADP, 5 mM $MgCl_2$, 20 mM HEPES (pH 7.8), and samples were sedimented at 100,000 rpm. (Beckman TL-100 Ultracentrifuge) at 20°C for 30 min. The amount of unbound S1 was determined by measuring the concentration of S1 in the supernatant after ultracentrifugation. The concentration of S1 was measured by a bicinchoninic acid method (BCA Protein Assay Reagent, Pierce Chemical Products, Rockford, IL).

Observation of fluorescently labeled actin filaments

Single actin filaments labeled with fluorescent phalloidin were observed with an inverted microscope (Nikon DIAPHOT-TMD) equipped with epifluorescence optics (Nikon, Tokyo, Japan), an objective (Zeiss Neofluar 100 X, oil immersion, NA 1.3), a 100W mercury arc lamp, and a Nikon rhodamine dichroic mirror and filter block. The fluorescence images were detected by a silicone-intensified-target camera (Hamamatsu C-2400, Hamamatsu Photonics, Japan) and stored in S-VHS format with a video cassette recorder (Panasonic NV-BS30S, Osaka, Japan). The sliding velocity of a single actin filament was quantified from the tape recordings using a computer image processor (Avio Excel; Nippon Avionics, Tokyo, Japan, and PC-9801DA; NEC, Tokyo, Japan) and custom software.

In vitro motility assay

To observe the movement of single actin filaments, HMM (3 to 5 μg) dissolved in 10 μl of a standard buffer solution (25 mM KCl, 5 mM $MgCl_2$ and 20 mM HEPES, pH 7.8) was placed on a coverslip (24 mm × 32 mm, Matsunami Glass Ind. Co., Osaka, Japan) that was pretreated with silicone (Sigmacote, Sigma Chemical Co., St. Louis, MO) and immediately covered with another smaller coverslip (18 mm × 18 mm), of which two diagonal edges were slightly upturned. After 90 s, 50 μl of the assay medium (see below) was applied from one edge, and the same volume of solution was collected from another edge. This procedure was repeated to wash out unbound HMM. The solution was then completely replaced by the assay medium containing: 0.05 M ionic strength (adjusted by KCl concentration), 5 mM MgCl2, 20 mM HEPES at various pHs, and various concentrations of the local anesthetics. To minimize photobleaching, an oxygen scavenging system (216 μg ml^{-1} glucose oxidase, 36 μg ml^{-1} catalase, 4.5 mg ml^{-1} glucose and 0.5% 2-mercaptoethanol) was added to the assay medium in all experiments (Harada et al., 1990). Three microliters of solution containing actin filaments (24 nM in actin monomers) labeled with PHDTMR were then applied to the coverslip, and the sliding movement started by adding assay medium containing 2 mM ATP. All experiments were performed at 30° ± 1°C. The data are presented as mean ± SD.

Local anesthetics

Two tertiary amine local anesthetics, lidocaine (2-diethylamino-*N*-[2,6-dimethylphenyl]acetamide) and tetracaine (4-[butylamino]benzoic acid 2-[dimethylamino]ethyl ester), and QX-314, a quaternary derivative of lidocaine (*N*-(2,6-dimethylphenylcarbamoylmethyl)-triethylammonium bromide) were used in this study. Lidocaine and tetracaine were supplied from Sigma Chemical Co. (St. Louis, MO): lidocaine crystalline, with pK_a of ~8.1 at 30°C, and tetracaine crystalline, with pK_a of ~8.5 at 30°C (Butterworth and Strichartz, 1990; Kamaya et al., 1983). The lidocaine derivative, QX-314, which has a permanent positive-charge, was supplied from Funakoshi Co. (Tokyo, Japan). The ratio of the charged form to the uncharged form of a tertiary amine local anesthetic is given by the following equation:

$$pK_a = pH + \log \frac{[BH^+]}{[B]} \quad (1)$$

where $[BH^+]$ and $[B]$ are the concentrations of charged and uncharged forms, respectively. Equation 1 indicates that the concentration of uncharged fraction increases as the pH value increases.

Force measurements

To measure the binding strength of actin filaments to myosin heads in the presence of anesthetic drugs, we combined an optical manipulation method with the in vitro motility assay system. The binding strength of actomyosin in the presence of anesthetics was measured by optical tweezers according to the methods of Block et al. (1990) and Ashkin et al. (1990). The optical tweezers apparatus and the method of determination of trapping force used have been described previously (Saito et al., 1994). One end of an actin filament was attached to a fluorescent latex bead (Polybead Microspheres, 1 μm in diameter, Polysciences, Inc., Warrington, PA), of which the surface had been previously coated with *N*-ethyl-maleimide-treated myosin to increase the affinity for actin (Kishino and Yanagida, 1988). The bead was trapped by an infrared laser (Nd:YAG-laser (1064 nm), Spectra-Physics Lasers, Inc., Mountain View, CA), and the other end was brought into contact with the HMM-coated surface (see Fig. 6). The binding strength was determined by measuring the breaking force of the actomyosin complex in assay medium containing 25 mM lidocaine. When the trapping force was sufficiently large, the actin filament was pulled from the HMM-coated surface by moving the stage with a mechanical micromanipulator (MHW-30, Narishige, Tokyo, Japan). As the trapping force was gradually decreased to a certain level, the breaking force exceeded the maximum trapping force and the bead was released from the trapping region. The breaking force was obtained from the maximum trapping force, which balanced just to the breaking force (Block et al., 1990; Ashkin et al., 1990).

The force of a single muscle fiber was measured by using a thin stainless steel wire. A single striated muscle fiber, which was obtained from rabbit psoas and skinned chemically by glycerol, was mounted on a stainless steel wire of ~0.2 mm in diameter. The force was determined by measuring the displacement of the wire by a photomultiplier.

RESULTS

Dose-dependent inhibition of sliding movement of single actin filaments by lidocaine and tetracaine

Movement of single actin filaments on an HMM-coated surface was observed in the presence and absence of lidocaine and tetracaine at pH 7.0 and 30°C. On the homogeneous surface of a coverslip treated with silicone, HMM were maximally packed in a single layer at a density of ~7.4 pmol mm^{-2} (Harada et al., 1990). Each sliding velocity was averaged from 20–30 different actin filaments in individual experiments and plotted as a function of the concentration of lidocaine and tetracaine, respectively. In the absence of anesthetics, the sliding velocity of single actin filaments on HMM was 10–12 $\mu m\ s^{-1}$, which was comparable with that in muscle fibers (Ishijima et al. 1996). In the presence of both lidocaine and tetracaine, the sliding movement was inhibited in a dose-dependent manner (Fig. 1), indicating the direct action of local anesthetics on the molecular motor proteins. Most of actin filaments (>95%) moved smoothly and continuously in the absence of anesthetics. As the concentration of the local anesthetics increased, the number of filaments that dissociated from the surface increased (see Fig. 7) and the number of filaments stationary on the surface increased. The percentage of stationary filaments was >80% of the filaments on the surface at 25 mM lidocaine or 2.5 mM tetracaine (Fig. 1). The velocities shown in Fig. 1 were measured only for filaments that moved continuously for >5 μm. The velocities decreased with increasing the concentrations of local anesthetics. Velocities slowed by the local anesthetics almost completely recovered to the original level (11 ± 0.7 $\mu m\ s^{-1}$ for lidocaine, 11 ± 0.8 $\mu m\ s^{-1}$ for tetracaine) when the anesthetics were removed from the assay medium. Dose-response curves yielded apparent inhibitory concentrations (IC_{50}), which gave a half maximum velocity of 17 mM for lidocaine and 2.5 mM for tetracaine. Tetracaine was ~7 times more effective in decreasing the sliding velocity than lidocaine.

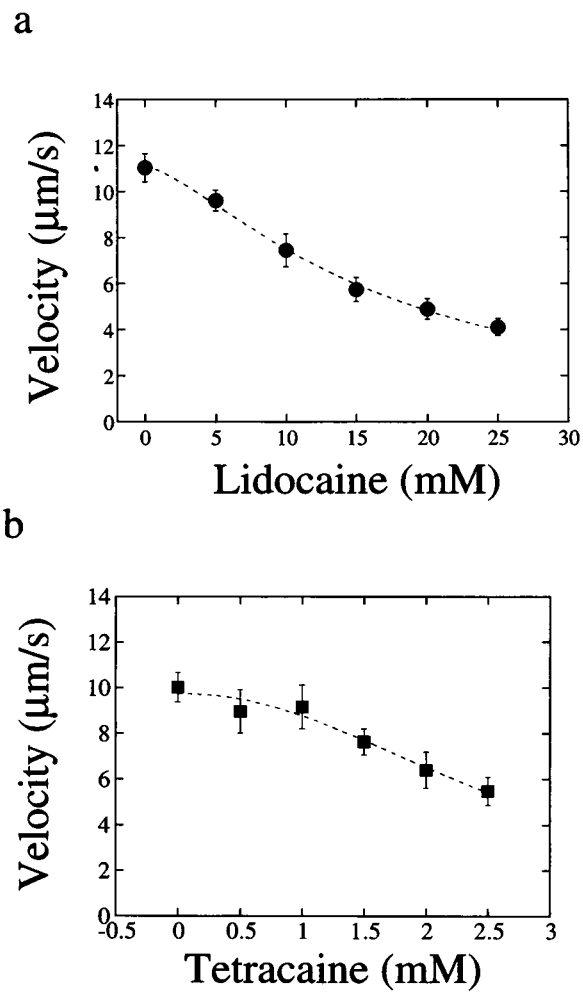

FIGURE 1 Inhibition of actin filament sliding velocity by lidocaine (*a*) and tetracaine (*b*). The sliding velocities of single actin filaments are plotted as a function of the total concentration of lidocaine (●) and tetracaine (■), respectively. Data points in the figures represent average sliding velocities of 20–30 different actin filaments, and the standard deviations are shown by vertical bars. The velocities were measured only for filaments that moved continuously more than 5 μm (see text). Assay medium: 5 mM $MgCl_2$, 2 mM ATP, 20 mM HEPES at pH 7.0 and at 0.05 M of the ionic strength (adjusted by KCl concentration). All experiments were performed at 30 ± 1°C. Declined velocities were almost completely recovered to the original levels (11 ± 0.7 m s^{-1} for lidocaine, 11 ± 0.8 m s^{-1} for tetracaine) by removal of anesthetic drugs.

Inhibition of force of a muscle fiber by an anesthetic

Fig. 2 shows the force of a single muscle fiber at various concentrations of lidocaine. In the standard in vitro assay solution (25 mM KCl), the force was minimally affected by 20 mM lidocaine. This is probably because actin and myosin molecules are very close to each other and actomyosin interaction is too strong to be affected by anesthetics at low ionic strength. Therefore, we measured the force at KCl concentration of 200 mM. The force decreased with increased concentrations of lidocaine and was almost completely inhibited at 20 mM. When lidocaine was washed out, the force recovered to the original level. Thus, the local anesthetic inhibits isometric force at the concentrations similar to those where the sliding movement is inhibited and its inhibitory action is reversible.

Effects of pH changes on the local anesthetic inhibition in actomyosin sliding movements

The relative amounts of protonated and unprotonated forms of tertiary amine local anesthetics depend on pH. Increasing

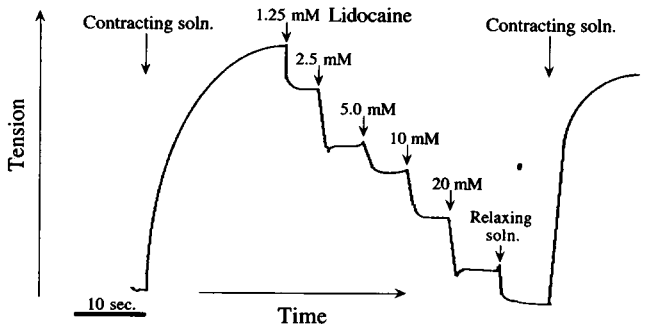

FIGURE 2 Inhibition of isometric force of a single muscle fiber by lidocaine. Assay medium: Relaxing solution, 200 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 2 mM HEPES at pH 7.0; Contracting solution, Relaxing solution + 2.5 mM $CaCl_2$. Arrows show replacement of solutions by those indicated. Numbers shown by mM unit indicate the concentrations of lidocaine in the Contracting solution. Temperature, 5°C.

the pH of the assay medium is expected to increase the fraction of uncharged forms according to Eq. 1, which explains the equilibrium between the two forms of local anesthetics (see Materials and Methods). To explore which form of the local anesthetic (charged or uncharged) predominantly affects the motor proteins, we measured the sliding velocities in the presence of local anesthetics at several pHs ranging from 6.5 to 8.5. The sliding movement of the actomyosin molecular motor has been found to be pH dependent (Kron and Spudich, 1986). In agreement with this, our results showed typical bell-shaped pH-dependence curves in the absence of local anesthetics (Fig. 3). The sliding velocities decreased in the presence of lidocaine (Fig. 3 *a*) and tetracaine (Fig. 3 *b*) at all tested pHs, and the extent of the decrease was greater at higher pHs with both drugs. For example, the extent of the decrease at pH 8.5 was ~3 times greater than that at pH 7.0 in the presence of 5 mM lidocaine. Therefore, this result strongly suggests that the inhibitory effect on the sliding movement is predominantly caused by the uncharged form of the local anesthetics because the increasing potency of local anesthetics at higher pH is accompanied by increasing fractions of the uncharged form.

To verify that the charged form of local anesthetics does not affect the motile function of actomyosin molecular motor, we tested the effect on sliding movements of QX-314, a permanently charged type of lidocaine derivative. The sliding velocity of actin filaments did not change in the presence of QX-314 at all the concentrations ranging from 0 to 20 mM (Fig. 4), indicating that the charged form of lidocaine had no effect on the actomyosin motor. This observation also suggests that the inhibitory action of local anesthetics on the actomyosin molecular motor is caused by the uncharged form.

Inhibitory action of uncharged local anesthetics on the sliding movement

To analyze the mode of action by the uncharged form of a local anesthetic in detail, we replotted the data in Fig. 3 as

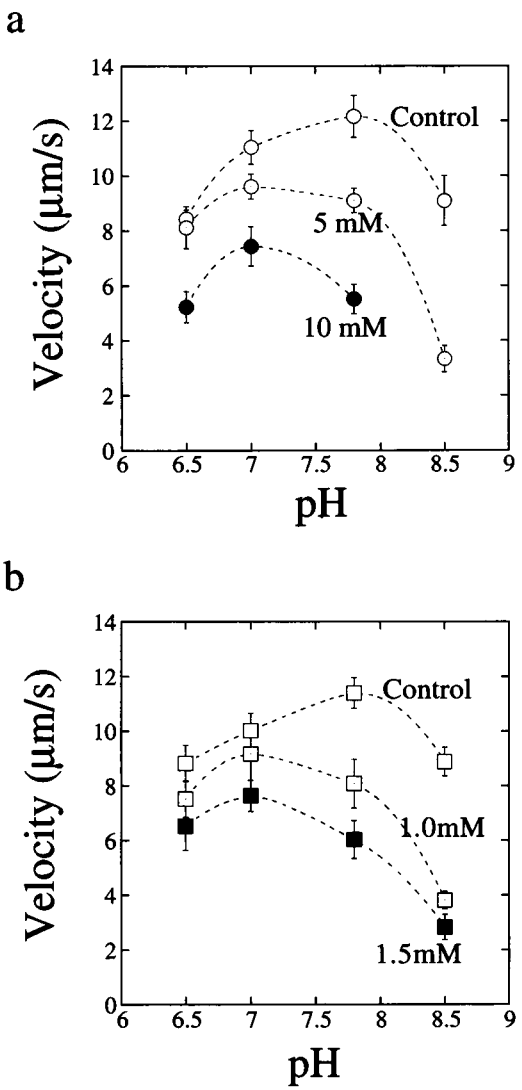

FIGURE 3 Effects of local anesthetics on the actin filament sliding movement at different pH values in the presence of lidocaine (*a*) and tetracaine (*b*). Data points in the figures indicate average sliding velocities of 20–30 different actin filaments, and the standard deviations are shown by vertical bars. Because the number of filaments stationary on the surface increased with increasing concentrations of the local anesthetics, the velocities were obtained for only the filaments that moved continuously more than 5 m. (*a*) Lidocaine. ○, 0 mM; ○, 5 mM; ●, 10 mM. (*b*) Tetracaine. □, 0 mM; □, 1.0 mM; ■, 1.5 mM. Assay medium was the same as that shown in Fig. 1, except for pH values. Temperature, 30 ± 1°C.

a function of the concentration of only the uncharged form of local anesthetics, considering the fractional changes of two forms accompanied with the pH changes (Fig. 5, *a* and *b*). We estimated the concentrations of the uncharged form of local anesthetics at various pHs according to Eq. 1 (refer to Materials and Methods). From the dose-response curves, the IC_{50} of the uncharged form was 0.76 mM at pH 6.5, 1.6 mM at pH 7.0, and 4.5 mM at pH 7.8 for lidocaine, and 24 μM at pH 6.5, 95 μM at pH 7.0, 170 μM at pH 7.8 and 450 μM at pH 8.5 for tetracaine. Data points in Fig. 3, *a* and *b* could be well fitted by the Hill equation with the Hill

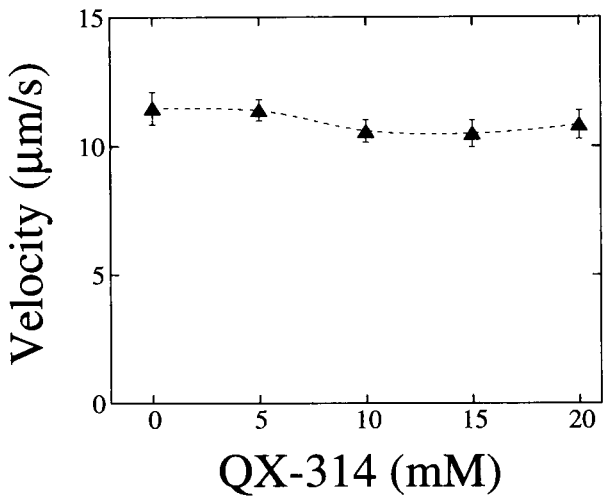

FIGURE 4 Effect of QX-314, a permanently charged quaternary derivative of lidocaine, on the actomyosin movement. The average sliding velocities of 20–30 actin filaments were plotted as a function of the concentration of QX-314. Vertical bars indicate the standard deviations.

coefficients of approximately two for both lidocaine and tetracaine, suggesting the mode of inhibitory action in the sliding movement was cooperative.

In light of the pH dependence of the dose-response curves, the inhibitory action by the uncharged form was much more enhanced as the pH decreased. This result suggests that the inhibitory action caused by the binding of uncharged anesthetic molecules may be linked with the preferential binding of some protons (Wyman, 1964).

Binding strength of an acto-HMM complex in the presence of local anesthetics

The velocity of actin filaments decreased with increasing concentrations of anesthetics. At saturating concentrations of local anesthetics, all of the actin filaments on the HMM-coated surface ceased to move, forming actomyosin complexes. To clarify the binding strength of HMM to actin, we measured the breaking force of the actomyosin complex in the assay medium containing 25 mM lidocaine, in which most of filaments on the surface remained stationary and the velocity of moving filaments decreased by ~70% (Fig. 1 *a*). The breaking force was ~1.5 pN per μm of an actin filament at saturating densities of HMM, which was >50-fold smaller than that of rigor complexes in the absence of ATP (Harada, personal communication) and ~10-fold smaller than active force in the absence of anesthetics (Ishijima et al., 1996). Thus, the binding of HMM to actin was weak in the presence of a local anesthetic.

We also examined whether actomyosin binding at the high concentration of an anesthetic was dependent on the ionic strength (Fig. 7). Most actin filaments remained bound to the surface at <10 mM KCl in the assay medium containing 25 mM lidocaine. The number of filaments bound to

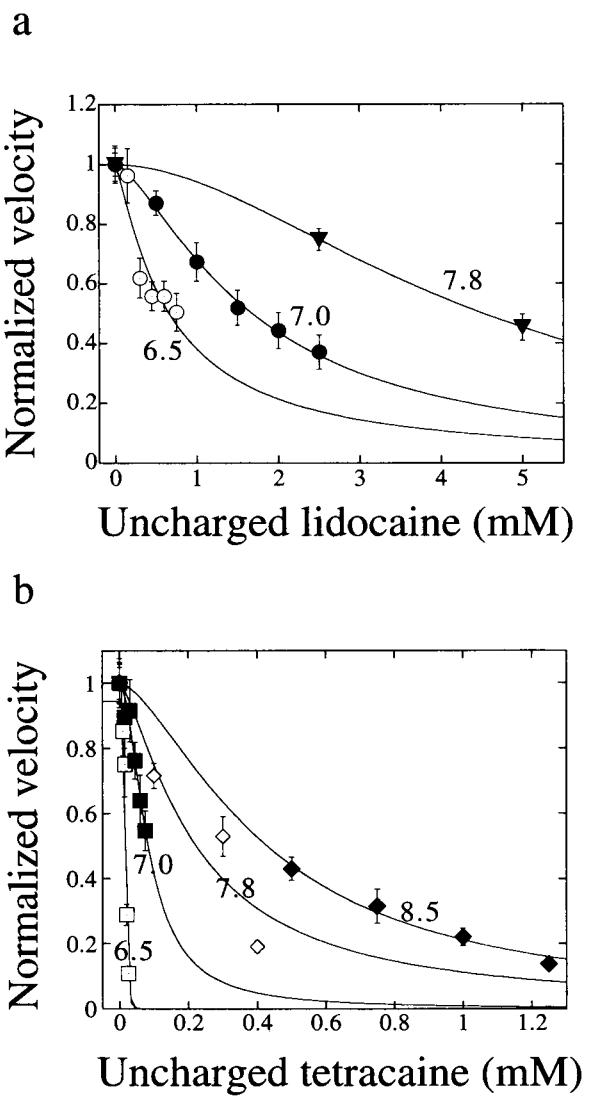

FIGURE 5 Effects of the uncharged forms of local anesthetics on the sliding velocity at various pH values. Data points at several pHs were replotted as a function of the concentration of the uncharged form of local anesthetics. The concentrations of the uncharged form of local anesthetics were calculated from Eq. 1 (see Methods). The velocities were normalized at each pH, taking the velocities of control as 1. The standard deviations are shown by vertical bars. (*a*) Inhibition by the uncharged lidocaine at pH 6.5 (○), pH 7.0 (●), and pH 7.8 (▼). (*b*) Tetracaine at pH 6.5 (□), pH 7.0 (■), pH 7.8 (◇), and pH 8.5 (◆). Solid lines indicate the theoretical curves derived from the best fitting to the Hill's theory by least mean squares method. These dose-response curves yielded the inhibitory concentrations of uncharged anesthetics (IC_{50}) of 0.76 mM at pH 6.5, 1.6 mM at pH 7.0, and 4.5 mM at pH 7.8 and of 24 M at pH 6.5, 95 M at pH 7.0, 170 M at pH 7.8, and 450 M at pH 8.5 for lidocaine and tetracaine, respectively.

the surface greatly decreased with increasing concentration of KCl (>10 mM, Fig. 7). In the absence of lidocaine, dependence of the binding of actin filament to HMM-coated surface on ionic strength was similar, but the binding was weakened at higher ionic strength. This is probably because one of the hydrophobic and ionic interactions that contribute to the acto-HMM binding, the hydrophobic interaction, is inhibited by lidocaine.

Effect of a local anesthetic on binding of acto-S1 complexes in the absence of ATP

In the absence of ATP, the binding of actin filaments to the HMM-coated surface was too strong to measure the binding even at high ionic strength. Therefore, we measured the binding of individual S1 molecules to actin filaments in solution. Fig. 8 shows the dissociation of S1 from F-actin in the absence of ATP but in the presence of ADP. In the absence of an anesthetic, S1 bound relatively rigidly to F-actin at low ionic strengths but mostly dissociated from F-actin at high ionic strengths. When lidocaine of 25 mM was added, most of S1 dissociated from F-actin even at low ionic strengths. Thus, lidocaine also binds to an acto-S1-ADP complex, which is expected to form an strong binding state, and weakens its binding greatly.

Effects of local anesthetics on the actin-activated myosin subfragment ATPase activity

Fig. 9 shows the V_{max} and K_m of actin-activated myosin subfragment ATPase in the presence of various concentrations of lidocaine (*a*) and tetracaine (*b*). The V_{max} values were slightly increased by the anesthetics, although the sliding velocity and force were significantly inhibited in these concentration ranges. The K_m values were increased approximately in parallel to the V_{max} values, which is consistent with dissociation of actin filaments from HMM on the surface (Fig. 7).

DISCUSSION

We have two purposes for examining the effect of local anesthetics on actomyosin. One is to examine whether the actomyosin motor proteins generally included in most living cells are affected by local anesthetics. For example, muscle containing the trigger area in the treatment of myofacial pain, into which local anesthetics are injected, is known to be relaxed. However, how local anesthetics affect the excitation-contraction coupling (E-C coupling) and contractile proteins has not been thoroughly examined . Another is to investigate the mode of action of local anesthetics on purified proteins. Actomyosin is advantageous for this purpose, because typical and important functions of proteins, enzymes, energy transduction, and molecular recognition are integrated in it. Here, we have demonstrated that local anesthetics directly affect purified motor proteins to inhibit the sliding movement of actin filaments in a reversible dose-dependent manner. This is probably the first reported account of the actions of local anesthetics on purified contractile proteins at the molecular level. The effective concentrations of the local anesthetics were millimolars (Figs. 1 and 2). The concentrations of local anesthetics injected into muscle containing the trigger area in the treatment of myofacial pain are generally tens of millimolars, so the effective concentrations of local anesthetics reported here are not so irrelevant to those for some clinical uses. The direct actions of local anesthetics on the actomyosin contractile proteins may modulate the intracellular organelle movement, the cell division, and the cell-shape formation.

The sliding velocity decreased with increasing concentrations of anesthetics, and most of actin filaments ceased to move and formed complexes with myosin bound to the surface at high concentrations (Figs. 1 and 4). If the anesthetic inhibits the actin-myosin interaction and causes complete dissociation of the complex, the velocity would not change in a dose-dependent manner, because unloaded velocity is independent of the number of intact myosin molecules that are involved in force generation, in the wide range of the number of myosin molecules (Harada et al., 1990). Therefore, the local anesthetics would not completely inhibit actomyosin interaction. The remaining interaction between actin and myosin would impose a load inhibiting actin filament sliding velocity.

Because the increasing potency of anesthetics at higher pH was accompanied by an increasing fraction of the uncharged form, it was suggested that the inhibitory effect of the sliding movement was predominantly caused by the uncharged form of the local anesthetic. The use of QX-314 gave a simple yet clear result that the charged type of lidocaine derivative was ineffective in reducing the sliding velocity. This result strongly supports the idea that the main active form of local anesthetics on the actomyosin molecular motor is the uncharged form. We conclude that the uncharged form of local anesthetics is primarily responsible to the inhibition of the motile function of actomyosin motor proteins. The local anesthetics would bind to hydrophobic regions of actomyosin interface and affect the hydrophobic interaction.

Biochemical studies have suggested that the force is generated by the conformational transition of actomyosin complex from a weakly binding to a strongly binding state (Eisenberg et al., 1985). Together the molecular model of the actomyosin complex based on the atomic structure of actin and myosin with the biochemical data, Holmes (1995) has suggested the sites for the weak and strong binding in the actomyosin interface. According to his argument, the weak binding is due to the interaction of the positively charged flexible loop (626th to 647th a.a.) of myosin motor domain with the negatively charged actin N-terminus. Thus, the weak binding is predominantly ionic. The strong binding includes hydrophobic and ionic interactions. The results showed that the uncharged form of local anesthetics predominantly inhibited the motility (Figs. 4 and 5), the breaking force of actomyosin at high concentrations of lidocaine was much smaller than that in rigor and the isometric force (Fig. 6), and the binding of actin filaments to myosin on the surface was hardly affected by the anesthetics at low ionic strength but was greatly weakened at higher ionic strength (Fig. 7). We can interpret from these results that the local anesthetics inhibit the hydrophobic interaction, which probably plays a major role of the strongly binding state, resulting in the inhibition of the sliding force generation. Meanwhile, the V_{max} of acto-S1 ATPase was not inhibited but

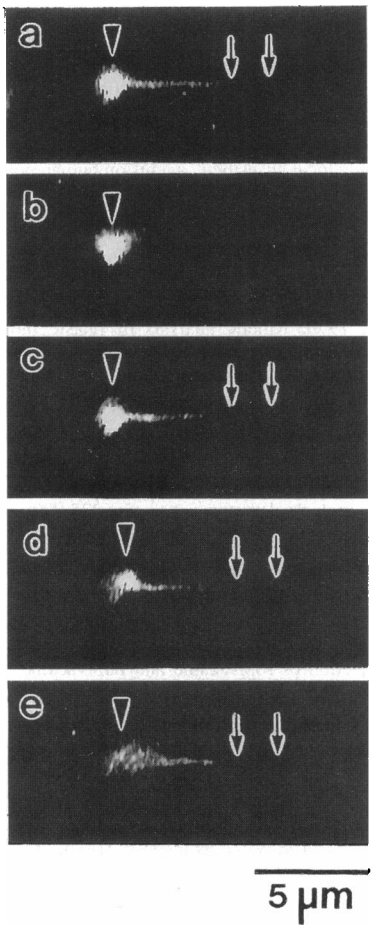

FIGURE 6 Measurement of the breaking force of acto-HMM complexes at 25 mM of lidocaine. One end of an actin filament was attached to a fluorescent bead trapped by the optical tweezers and another end was brought into contact with the HMM-coated surface. Arrow heads indicate the fluorescent images of the bead and arrows indicate the segment of actin filament that interacts with the HMM-coated surface. When the trapping force exceeded the strength of actomyosin complex, the actin filament was removed from the surface by moving the stage (*b*). The trapping force was gradually decreased by reducing the laser power (*c–e*). When the trapping force was decreased to a certain level, the breaking force exceeded the maximum trapping force at a certain trapping force and the bead was released from the trapping region (*e*). The breaking force was obtained from the trapping force, which balanced just to the strength of acto-HMM complex.

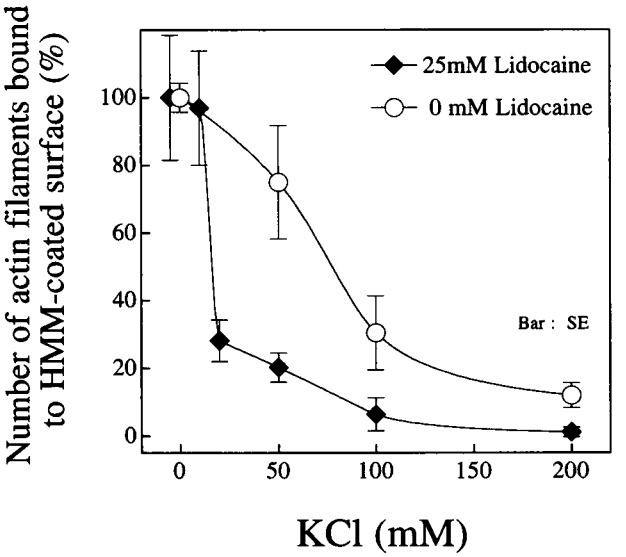

FIGURE 7 Effects of ionic strength on the binding actin filaments to the HMM-coated surface in the presence of lidocaine. The number of actin filaments bound to HMM-coated surface of 50 frames was counted in solution containing 5 mM $MgCl_2$, 2 mM ATP, 20 mM HEPES at pH 7.8, and KCl of concentrations indicated in the abscissa. ○ and ◆, 0 and 25 mM lidocaine, respectively. The number of actin filaments was normalized, taking the number in the absence of ATP as 100%. Each vertical bar represents the standard deviations.

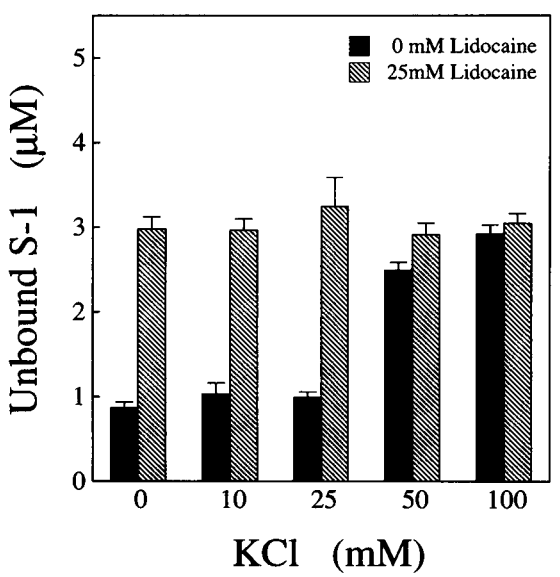

FIGURE 8 Effect of lidocaine on binding of acto-S1 complexes in the absence of ATP. Binding of S1 (5 μM) to actin filaments (10 μM in monomer) was measured in solution containing 0–100 mM KCl, 2 mM ADP, 5 mM $MgCl_2$, 20 mM HEPES (pH 7.8), and 0 (■) or 25 mM (▨) lidocaine. Vertical bars show the standard deviations (n = 6).

slightly increased by anesthetics (Fig. 9). Although the K_m values were also increased approximately in parallel, the actin-activated ATPase activities of HMM on the surface or myosin in muscle fibers are probably high in the presence of anesthetics, because the effective concentration of actin for HMM or myosin in the vicinity of actin filaments on the surface or in muscle fibers would be relatively high. Therefore, decreases in the sliding velocity and the force of muscle fibers in the presence of anesthetics are not due to a decrease in the actin-activated HMM or myosin ATPase activity. Thus, the results suggest that the binding and ATPase of actomyosin are governed predominantly by ionic weak and strong binding, which are hardly affected by local anesthetics; and the force generation requires hydrophobic interaction, which plays a major part of the strong binding and is blocked by the local anesthetics, in addition to the ionic binding. To lead to more rigid conclusion, it is indispensable to develop new methods. Recently, we have de-

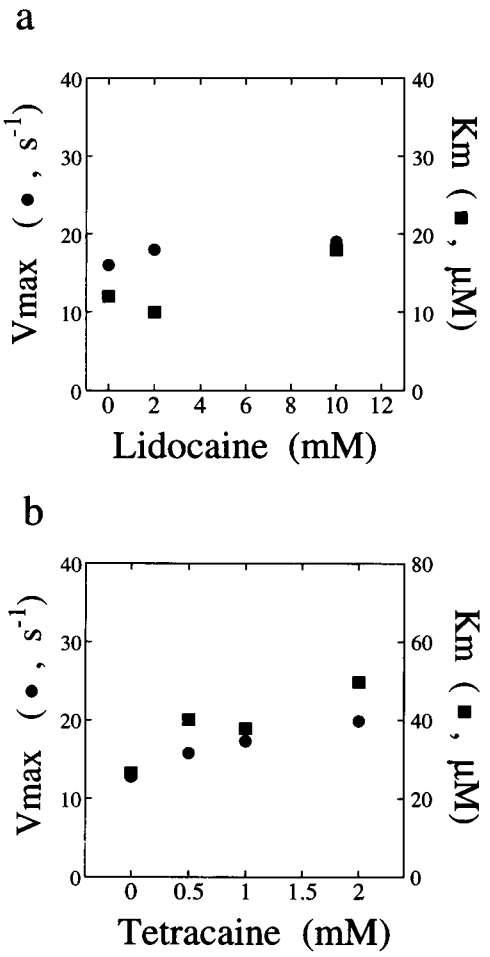

FIGURE 9 Actin-activated ATPase activities of myosin subfragments in the presence of lidocaine or tetracaine. The ATPase activity was measured in the standard assay buffer containing various concentrations of anesthetics at 20°C. HMM and S1 were used for lidocaine (*a*) and tetracaine (*b*), respectively. The values of V_{max} and K_m were determined by the Lineweaver-Burk plots.

veloped a novel method by which we can directly measure the interacting force between actin and myosin at the single molecular level, controlling their gap with nanometer accuracy (Tokunaga and Yanagida, unpublished data). This method will enable us to measure the ionic and hydrophobic interactions between actin and myosin molecules and more directly show how these interactions are affected by anesthetics.

We thank Prof. T. Kodama (Kyushu Inst. Tech.) for critical reading of this manuscript and variable discussion.

REFERENCES

Ashkin, A., K. Schütze, J. M. Dziedzic, U. Euteneuer, and M. Schliwa. 1990. Force generation of organelle transport measured in vivo by an infrared laser trap. *Nature (Lond.).* 348:346–348.

Block, S. M., L. S. B. Goldstein, and B. J. Schnapp. 1990. Bead movement by single kinesin molecules studied with optical tweezers. *Nature (Lond.).* 348:348–352.

Brenner, B. 1991. Rapid dissociation and reassociation of actomyosin cross-bridges during force generation: a newly observed facet of cross-bridge action in muscle. *Proc. Natl. Acad. Sci. USA.* 88:10490–10494.

Butterworth, IV, J. F., and G. R. Strichartz. 1990. Molecular mechanisms of local anesthesia: a review. *Anesthesiology.* 72:711–734.

Cederholm, I., G. Briheim, H. Rutberg, and C. Dahlgren. 1994. Effects of five amino-amide local anaesthetic agents on human polymorphonuclear leukocytes measured by chemiluminescence. *Acta. Anaesthesiol. Scand.* 38:704–710.

Chernoff, D. M., and G. R. Strichartz. 1989. Tonic and phasic block of neuronal sodium currents by 5-Hydroxyhexano-2′,6′-xylide, a neutral lidocaine homologue. *J. Gen. Physiol.* 93:1075–1090.

Chernoff, D. M. 1990. Kinetic analysis of phasic inhibition neuronal sodium currents by lidocaine and bupivacaine. *Biophys. J.* 58:53–68.

Courtney, K. R. 1986. Potentially toxic actions of local anesthetics in cardiac tissue. *In* Molecular and Cellular Mechanisms of Anesthetics. S. H. Roth and K. W. Miller, editors. Plenum Medical Book Company, New York and London. 377–384.

Eisenberg, E., and T. L. Hill. 1985. Muscle contraction and free energy transduction in biological systems. *Science.* 227:999–1006.

Elliott, J. R., and D. A. Haydon. 1989. The actions of neutral anaesthetics on ion conductances of nerve membranes. *Biochim. Biophys. Acta.* 988:257–286.

Feldman, H. S., G. R. Arthur, M. Pitkanen, R. Hurley, A. M. Doucette, and B. G. Covino. 1991. Treatment of acute systemic toxicity after the rapid intravenous injection of ropivacaine and bupivacaine in the conscious dog. *Anesth. Analg.* 73:373–384.

Harada, Y., A. Noguchi, A. Kishino, and T. Yanagida. 1987. Sliding movement of single actin filaments on one-headed myosin filaments. *Nature (Lond.).* 326:805–808.

Harada, Y., K. Sakurada, T. Aoki, D. D. Thomas, and T. Yanagida. 1990. Mechanochemical coupling in actomyosin energy transduction studied by in vitro movement assay. *J. Mol. Biol.* 216:49–68.

Hille, B. 1977. Local anesthetics: hydrophilic and hydrophobic pathways for the drug-receptor reaction. *J. Gen. Physiol.* 69:497–515.

Holmes, C. K. 1995. The actomyosin interaction and its control by tropomyosin. *Biophys. J.* 68:2s–7s.

Homsher, E., F. Wang, and J. R. Sellers. 1992. Factors affecting movement of F-actin filaments propelled by skeletal muscle heavy meromyosin. *Am. J. Physiol.* 262:c714–c723.

Huxley, H. A. 1990. Sliding filaments and molecular motile systems. *J. Biol. Chem.* 265:8347–8350.

Ishijima, A., H. Kojima, H. Higuchi, Y. Harada, T. Funatsu, and T. Yanagida. 1996. Multiple-and single-molecule analysis of the actomyosin motor using nanometer-piconewton manipulation with a microneedle: unitary steps and forces. *Biophys. J.* 70:383–400.

Kamaya, H., J. J. Hayes, Jr., and I. Ueda. 1983. Dissociation constants of local anesthetics and their temperature dependence. *Anesth. Analg.* 62: 1025–1030.

Kishino, A., and T. Yanagida. 1988. Force measurements by micromanipulation of a single actin filament by glass needles. *Nature (Lond.).* 334:74–76.

Kron, S. J., and J. A. Spudich. 1986. Fluorescent actin filaments move on myosin fixed to a glass surface. *Proc. Natl. Acad. Sci. USA.* 83: 6272–6276.

Margossian, S. S., S. Lowey, and B. Barshop. 1982. Preparation of myosin and its subfragments from rabbit skeletal muscle. *Methods Enzymol.* 85:55–71.

Moller, R. A., and B. G. Covino. 1992. Effect of progesterone on the cardiac electrophysiologic alterations produced by ropivacaine and bupivacaine. *Anesthesiology.* 77:735–741.

Narahashi, T., D. T. Frazier, and M. Yamada. 1970. The site of action and active form of local anesthetics. *J. Pharmacol. Exp. Ther.* 171:32–51.

Ragsdale, D. S., J. C. McPhee, T. Scheuer, and W. A. Catterall. 1994. Molecular determinants of state-dependent block of Na^+ channels by local anesthetics. *Science.* 265:1724–1728.

Reiz, S., and S. Nath. 1986. Cardiotoxicity of local anaesthetic agents. *Br. J. Anaesth.* 58:736–746.

Rosenberg, P. H., J. Zou, and J. E. Heavner. 1993. Comparison of acute central nervous system and cardiovascular toxicity of 2-chloroprocaine and prilocaine in the rat. *Acta Anaesthesiol. Scand.* 37:751–755.

Saito, K., T. Aoki, T. Aoki, and T. Yanagida. 1994. Movement of single myosin filaments and myosin step size on an actin filament suspended in solution by a laser trap. *Biophys. J.* 66:769–777.

Sasagawa, S. 1991. Inhibitory effects of local anesthetics on migration, extracellular release of lysosomal enzyme, and superoxide anion production in human polymorphonuclear leukocytes. Immunopharmacol. *Immunotoxicology.* 13:607–622.

Schneider, M. F., and J.-M. Dubois. 1986. Effects of benzocaine on the kinetics of normal and batrachotoxin-modified Na^+ channels in frog node of ranvier. *Biophys. J.* 50:523–530.

Schwarz, W., P. T. Palade, and B. Hille. 1977. Local anesthetics: effect of pH on use-dependent block of sodium channels in frog muscle. *Biophys. J.* 20:343–368.

Takagi, S., S. Kitagawa, K. Oshimi, F. Takaku, and Y. Miura. 1983. Effect of local anaesthetics on human natural killer cell activity. *Clin. Exp. Immunol.* 53:477–481.

Toyoshima, Y. Y., S. J. Kron, E. M. McNally, K. R. Niebling, C. Toyoshima, and J. A. Spudich. 1987. Myosin subfragment-1 is sufficient to move actin filaments in vitro. *Nature (Lond.).* 328:536–539.

Vandam, L. D. 1987. Some aspects of the history of local anesthesia, local anesthetics. *In* Handbook of Experimental Pharmacology. G. R. Strichartz, editor. Springer-Verlag, Heidelberg. 1–19.

Wang, G. K., W. M. Mok, and S.-Y. Wang. 1994. Charged tetracaine as an inactivation enhancer in batrachotoxin-modified Na^+ channels. *Biophys. J.* 67:1851–1860.

Wasserstrom, J. A., K. Liberty, J. Kelly, P. Santucci, and M. K. Myers. 1993. Modification of cardiac Na^+ channels by batrachotoxin: effects on gating, kinetics, and local anesthetic binding. *Biophys. J.* 65: 386–395.

Weeds, A. G., and R. S. Taylor. 1982. Preparation of myosin and its subfragment from rabbit skeletal muscle. *Methods Enzymol.* 85:55–71.

Wyman, J. 1964. Linked functions and reciprocal effects in hemoglobin: a second look. *Advan. Protein Chem.* 19:223–286.

Yanagida, T., and F. Oosawa. 1978. Polarized fluorescence from ϵ-ADP incorporated into F-actin in a myosin-free single fiber: conformation of F-actin and changes induced in it by heavy meromyosin. *J. Mol. Biol.* 126:507–524.

Yanagida, T., I. Kuranaga, and A. Inoue. 1982. Interaction of myosin with thin filaments during contraction and relaxation: effect of ionic strength. *J. Biochem.* 92:407–412.

Yanagida, T., M. Nakase, K. Nishiyama, and F. Oosawa. 1984. Direct observation of motion of single F-actin filaments in the presence of myosin. *Nature (Lond.).* 307:58–60.

Zamponi, G. W., and R. J. French. 1993. Dissecting lidocaine action: diethylamide and phenol mimic separate modes of lidocaine block of sodium channels from heart and skeletal muscle. *Biophys. J.* 65: 2335–2347.