# Lidocaine Preferentially Inhibits the Function of Purinergic P2X7 Receptors Expressed in *Xenopus* Oocytes

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**BACKGROUND:** Lidocaine has been widely used to relieve acute pain and chronic refractory pain effectively by both systemic and local administration. Numerous studies reported that lidocaine affects several pain signaling pathways as well as voltage-gated sodium channels, suggesting the existence of multiple mechanisms underlying pain relief by lidocaine. Some extracellular adenosine triphosphate (ATP) receptor subunits are thought to play a role in chronic pain mechanisms, but there have been few studies on the effects of lidocaine on ATP receptors. We studied the effects of lidocaine on purinergic P2X3, P2X4, and P2X7 receptors to explore the mechanisms underlying pain-relieving effects of lidocaine.

**METHODS:** We investigated the effects of lidocaine on ATP-induced currents in ATP receptor subunits, P2X3, P2X4, and P2X7 expressed in *Xenopus* oocytes, by using whole-cell, two-electrode, voltage-clamp techniques.

**RESULTS:** Lidocaine inhibited ATP-induced currents in P2X7, but not in P2X3 or P2X4 subunits, in a concentration-dependent manner. The half maximal inhibitory concentration for lidocaine inhibition was 282 ± 45 μmol/L. By contrast, mepivacaine, ropivacaine, and bupivacaine exerted only limited effects on the P2X7 receptor. Lidocaine inhibited the ATP concentration–response curve for the P2X7 receptor via noncompetitive inhibition. Intracellular and extracellular *N*-(2,6-dimethylphenylcarbamoylmethyl) triethylammonium bromide (QX-314) and benzocaine suppressed ATP-induced currents in the P2X7 receptor in a concentration-dependent manner. In addition, repetitive ATP treatments at 5-minute intervals in the continuous presence of lidocaine revealed that lidocaine inhibition was use-dependent. Finally, the selective P2X7 receptor antagonists Brilliant Blue G and AZ11645373 did not affect the inhibitory actions of lidocaine on the P2X7 receptor.

**CONCLUSIONS:** Lidocaine selectively inhibited the function of the P2X7 receptor expressed in *Xenopus* oocytes. This effect may be caused by acting on sites in the ion channel pore both extracellularly and intracellularly. These results help to understand the mechanisms underlying the analgesic effects of lidocaine when it is administered locally at least. (Anesth Analg 2015;120:597–605)

Systemic administration of lidocaine has been used to relieve neuropathic pain, including that from malignant and nonmalignant disorders.[1,2] Abnormal expression of voltage-gated sodium channels in both injured and neighboring areas correlates with ectopic activity[3] that has been proposed as a mechanism underlying neuropathic pain,[4] suggesting the role of blockade of specific sodium channels by intravenous lidocaine to produce neuropathic pain relief.[5] However, lidocaine has other several beneficial effects in some clinical situations such as postoperative pain relief by epidural administration, stimulation of bowel function after colon surgery,[6] and topical anesthesia in addition to properties including antithrombotic[7] and anti-inflammatory actions,[8] which are potentially important during the perioperative period. These multiple effects of lidocaine indicate that there may be mechanisms other than sodium channel blockade. Moreover, the difference of effective concentrations between intravenous and epidural administration also supports this possibility. Indeed, several reports demonstrated that lidocaine affects other pain signaling pathways,[9] receptors, and ion channels including Gq protein,[10] potassium channels,[11] and calcium channels.[12]

Extracellular adenosine triphosphate (ATP) is a neurotransmitter acting through ATP receptors, which are classified as ligand-gated ion channels (P2X receptors) and G-protein–coupled receptors (P2Y receptors). Seven P2X (P2X1–7) and eight P2Y (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11–14) subunits have been identified.[13] They are distributed in multiple organs and have important roles in various physiologic functions.[14,15] Recently, specific receptor subunits have been shown to be involved in various pathologic conditions, including brain ischemia, pain, inflammation,

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osteoporosis, spinal cord injury, and bladder dysfunction; therefore, these receptors are considered to be potential therapeutic targets.[16–19] It is especially suggested that several subunits including P2X3, P2X4, and P2X7 receptors, which are distributed in pain pathways within the nervous system, are involved in chronic pain mechanisms.[20]

P2X3 receptors, which are mainly distributed in sensory neurons such as dorsal root ganglia, have been shown to be involved in the mechanism of neuropathic pain by demonstrating that intrathecal treatment with P2X3 antisense oligonucleotide decreased nociceptive behavior in a model of chronic inflammation and reduced mechanical allodynia in a rat model of neuropathic pain.[21] P2X4 receptors are widely expressed in the brain, spinal cord, autonomic and sensory ganglia, and microglia. Several reports demonstrated that upregulation of P2X4 receptors in activated microglia located in the dorsal horn of the spinal cord contributes to neuropathic pain.[22] P2X7 receptors are expressed on cells of the immune system as well as glial cells. In addition, inflammatory and neuropathic hypersensitivities in response to mechanical and thermal stimuli were completely absent in mice lacking P2X7 receptors,[23] suggesting a role of P2X7 receptors in pain modulation.

Although previous reports have demonstrated the effects of some general anesthetics, ethanol, and antidepressants on P2X receptors,[24–28] no studies have investigated whether local anesthetics act on these receptors. Therefore, we investigated the effects of lidocaine and other local anesthetics on P2X3, P2X4, and P2X7 receptors to explore the mechanisms underlying the pain-relieving effects of lidocaine.

## METHODS

This study was approved by the Animal Research Committee of the University of Occupational and Environmental Health, Kitakyushu, Japan.

### Drugs

All chemicals, including ATP disodium salt, lidocaine hydrochloride, ropivacaine hydrochloride, bupivacaine hydrochloride, benzocaine, *N*-(2,6-dimethylphenylcarbamoylmethyl) triethylammonium bromide (QX-314), AZ11645373, and Brilliant Blue G (BBG), were purchased from Sigma-Aldrich (St. Louis, MO).

### Plasmids

All plasmids including human P2X3, P2X4, and P2X7 receptor complementary DNA (cDNA) were purchased from OriGene Technologies (Rockville, MD).

### cRNA Preparation and Oocyte Injection

After double digestion of cDNA with *Sac*I and *Sma*I (P2X3 receptor) or linearization with *Sac*I (P2X4 and P2X7 receptors), complementary RNAs (cRNA) were transcribed using T7 RNA polymerase using an mMESSAGE mMACHINE kit (Ambion, Austin, TX). Adult female *Xenopus laevis* frogs were obtained from Kyudo (Saga, Japan). *X. laevis* oocytes and cRNA microinjection were prepared and performed as described previously.[29,30] In brief, stage IV to VI oocytes were manually isolated from a removed portion of ovary. Next, oocytes were treated with collagenase (0.5 mg/mL) for 10 minutes and placed in modified Barth solution (88 mmol/L NaCl, 1 mmol/L KCl, 2.4 mmol/L $NaHCO_3$, 10 mmol/L HEPES, 0.82 mmol/L $MgSO_4$, 0.33 mmol/L $Ca[NO_3]_2$, and 0.91 mmol/L $CaCl_2$, adjusted to pH 7.5), supplemented with 10,000 U penicillin, 50 mg gentamicin, 90 mg theophylline, and 220 mg/L sodium pyruvate (incubation medium). cRNAs of P2X receptors were injected (total volume was 0.5–20 ng/50 nL) into *Xenopus* oocytes. Injected oocytes were incubated at 19°C in incubation medium, and 2 to 6 days after injection, the cells were used for electrophysiologic recordings.

### Electrophysiologic Recordings

All electrical recordings were performed at room temperature (20–23°C). Oocytes were placed in a 100-μL recording chamber and perfused at 2 mL/min with extracellular Ringer's solution (110 mmol/L NaCl, 2.5 mmol/L KCl, 10 mmol/L HEPES, 1.8 mmol/L $BaCl_2$, pH 7.5) using a peristaltic pump (World Precision Instruments, Sarasota, FL). $Ca^{2+}$ in the solution was replaced with $Ba^{2+}$ to prevent the activation of $Ca^{2+}$-dependent $Cl^-$ channels.[31,32] Recording electrodes were prepared with borosilicate glass capillary tubing using a puller (PP-830; Narishige, Tokyo, Japan); microelectrodes had a resistance of 1 to 3 MΩ when filled with 3 mol/L KCl. Whole-cell voltage clamp was achieved using these two electrodes with a Warner Instruments model OC-725C system (Warner, Hamden, CT) at −70 mV. Local anesthetics, BBG, and ATP disodium salt stocks were prepared and diluted before adding to the bath solution. AZ11645373 stocks were prepared in dimethylsulfoxide and diluted in bath solution to a final dimethylsulfoxide concentration not exceeding 0.05%. We measured the peak of the transient inward current in response to ATP that was applied for 20 seconds. Local anesthetics were preapplied for 2 minutes to allow a complete change of bath solution.

To characterize the inhibitory effect of lidocaine on P2X7 receptors, we measured ATP-induced currents at 10 μmol/L to 5 mmol/L in the presence or absence of 300 μmol/L lidocaine. The quaternary local anesthetic QX-314, which is 99.9% permanently charged and does not penetrate the cell membrane, was either injected directly into oocytes or applied outside the cell to identify whether it acts intracellularly or extracellularly. QX-314 (50 nL of 5 mmol/L diluted in 150 mmol/L KCl) was injected into oocytes to result in an intracellular concentration of approximately 500 μmol/L although, in practice, the intracellular concentration in each oocyte may be variable because the oocytes are heavily compartmentalized. Control cells were injected with 150 mmol/L KCl. Recordings were performed 10 minutes after injection. To investigate the potential use-dependent effects of lidocaine, ATP was applied at 5-minute intervals in the continuous presence of 100 μmol/L lidocaine for 30 minutes. The results are expressed as percentages of control responses.

### Statistical Analyses

GraphPad Prism software (GraphPad Software, San Diego, CA) was used to conduct the statistical analysis. All values are presented as means ± SEM. The *n* values refer to the number of oocytes examined. Each experiment was performed with oocytes from at least two frogs.

The concentration–response curves for the ATP-induced peak current were fitted using the logistic function: $I = I_{min} + (I_{max} - I_{min})/[1 + (EC_{50}/A)^n]$, where $I$ is the response induced by ATP concentration, $I_{min}$ is the minimal response, $I_{max}$ is the maximal response, $EC_{50}$ is the half maximal effective concentration, $A$ is the concentration of ATP, and $n$ is the Hill coefficient. Differences were evaluated statistically using unpaired, two-tailed $t$ test and one-way analysis of variance followed by Dunnett multiple comparison test, where the objective is to identify groups whose means are significantly different from the mean of a selected "reference group," in our case, no treatment with local anesthetics, or Tukey multiple comparison test, where the mean of each group is compared with the mean of every other group. Hill coefficient, half maximal inhibitory concentration ($IC_{50}$), and $EC_{50}$ values were also calculated. Values of $P < 0.01$ were taken as showing a significant difference.

## RESULTS

### Effects of Lidocaine on Peak ATP-Induced Inward Currents in the P2X3, P2X4, and P2X7 Receptors

We determined the ATP concentration–response relation under our experimental conditions for P2X3, P2X4, and P2X7 receptors. Nonlinear regression analyses of the curves yielded the $EC_{50}$ for ATP and slope variables (Hill coefficient) of 2.3 ± 0.8 μmol/L and 0.74 ± 0.1 in oocytes expressing P2X3 receptors, 10.8 ± 3.3 μmol/L and 0.69 ± 0.2 in oocytes expressing P2X4 receptors, and 1.2 ± 0.1 mmol/L and 3.7 ± 0.9 in oocytes expressing P2X7 receptors, respectively (Fig. 1). Based on these results, the effects of lidocaine on ATP-induced currents were examined at an ATP concentration of 2 μmol/L for P2X3 receptor, 10 μmol/L for P2X4 receptor, and 1 mmol/L for P2X7 receptor. Figure 2B shows concentration–response relations of lidocaine-mediated inhibition on ATP-induced currents in three receptors. Lidocaine inhibited the currents in a concentration-dependent manner in oocytes expressing P2X7 receptor; the $IC_{50}$ value of lidocaine for ATP-induced currents and the slope were 282 ± 45 μmol/L and 0.72 ± 0.07, respectively (Table 1). These inhibitory effects were significant at concentrations of lidocaine ≥30 μmol/L (Fig. 2B). By contrast, the inhibitory effects of lidocaine on the P2X3 and P2X4 receptors were limited. Specifically, only a high concentration of 10 mmol/L lidocaine significantly suppressed ATP-induced currents to 68 ± 6% and 71 ± 6% of the control in oocytes expressing P2X3 and P2X4 receptors, respectively (Fig. 2B).

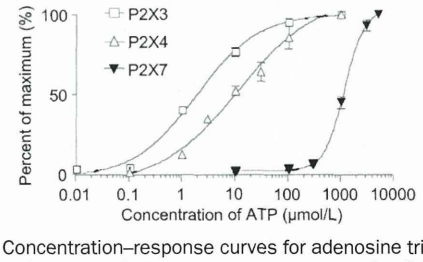


**Figure 1.** Concentration–response curves for adenosine triphosphate (ATP)-induced currents in *Xenopus* oocytes expressing P2X3, P2X4, and P2X7 receptors. Oocytes were voltage clamped at –70 mV. ATP (10 nmol/L to 1 mmol/L for P2X3; 100 nmol/L to 1 mmol/L for P2X4; and 10 μmol/L to 5 mmol/L for P2X7) was applied for 20 seconds, and peak current was measured. Values are the mean ± SEM ($n$ = 6).

### Effects of Other Local Anesthetics on the P2X7 Receptor

We next examined the effects of other local anesthetics including mepivacaine, ropivacaine, and bupivacaine on ATP-induced currents in oocytes expressing P2X7 receptor because lidocaine inhibited this receptor most strongly. Although mepivacaine suppressed ATP-induced currents in a concentration-dependent manner, the inhibitory effects were less than those of lidocaine with the $IC_{50}$ value and slope variable of 6.0 ± 0.06 mmol/L and 0.62 ± 0.06, respectively. This suggests that the inhibitory potency of mepivacaine was one-twentieth of that of lidocaine (Fig. 3, Table 1). By contrast, ropivacaine and bupivacaine had little effect on ATP-induced currents (Fig. 3).

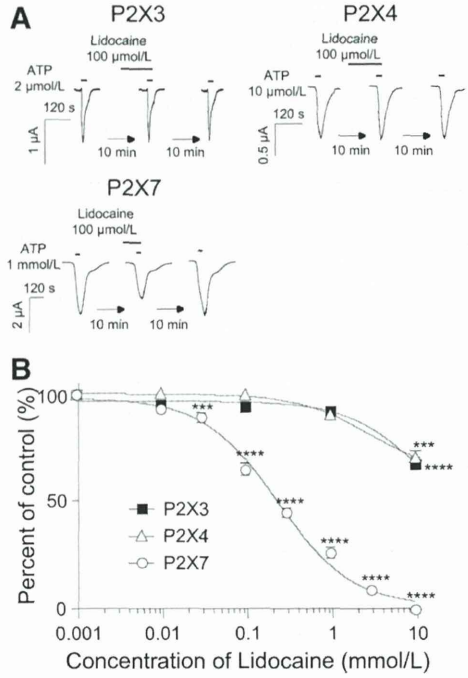


**Figure 2.** Effects of lidocaine on the adenosine triphosphate (ATP)-induced currents in *Xenopus* oocytes expressing P2X3, P2X4, and P2X7 receptors. A, Representative traces from oocytes expressing P2X3, P2X4, and P2X7 receptors in both the absence and presence of 100 μmol/L lidocaine. P2X3, P2X4, and P2X7 receptors were activated by 2 μmol/L, 10 μmol/L, and 1 mmol/L of ATP, respectively. ATP was applied for 20 seconds with or without pretreatment for 2 minutes with 100 μmol/L lidocaine. The currents of lidocaine-treated oocytes were recorded 10 minutes after recording the control currents, and the washout currents were obtained 10 minutes after lidocaine treatment. B, Concentration–response curves for the inhibitory effects of lidocaine on ATP-induced currents in P2X3, P2X4, and P2X7 receptors. The peak current amplitude in the presence of lidocaine was normalized to that of the control and the effects are expressed as percentages of the control. Lidocaine inhibited ATP-induced currents in oocytes expressing both P2X3 and P2X4 receptors at only a high concentration of 10 mmol/L. In contrast, lidocaine suppressed ATP-induced currents in oocytes expressing P2X7 concentration dependently, and it reduced those currents to 93 ± 4%, 89 ± 5%, 65 ± 8%, 45 ± 6%, 26 ± 7%, and 9 ± 2% of control at 10 μmol/L, 30 μmol/L, 100 μmol/L, 300 μmol/L, 1 mmol/L, and 3 mmol/L, respectively. Data are presented as means ± SEM ($n$ = 6). Hill coefficient and half maximal inhibitory concentration ($IC_{50}$) values were calculated using GraphPad Prism. ***$P < 0.001$ and ****$P < 0.0001$ compared with the control (one-way analysis of variance followed by Dunnett post hoc test).

**Table 1. Effects of Lidocaine, Mepivacaine, QX-314, and Benzocaine on $IC_{50}$ Value and Hill Coefficient Calculated from the Concentration–Response Curves Shown in Figures 3 and 5**

| | Lidocaine | Mepivacaine | QX-314 | Benzocaine |
|---|---|---|---|---|
| $IC_{50}$ (μmol/L) | 282 ± 45 | 6020 ± 61 | 500 ± 75 | 1560 ± 39 |
| Hill coefficient | 0.72 ± 0.07 | 0.62 ± 0.06 | 0.36 ± 0.03 | 0.88 ± 0.07 |

$IC_{50}$ = half maximal inhibitory concentration; QX-314 = (*N*-[2,6-dimethylphenylcarbamoylmethyl] triethylammonium bromide).

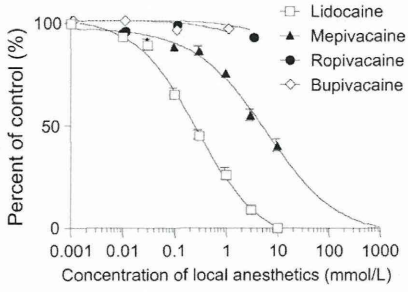


**Figure 3.** Concentration–response curves for inhibitory effects of local anesthetics including lidocaine, mepivacaine, ropivacaine, and bupivacaine on adenosine triphosphate–induced currents in P2X7 receptors. The peak current amplitude in the presence of local anesthetics was normalized to that of the control and the effects are expressed as percentages of the control. Data are presented as means ± SEM ($n$ = 6). Hill coefficient and half maximal inhibitory concentration ($IC_{50}$) values were calculated using GraphPad Prism.

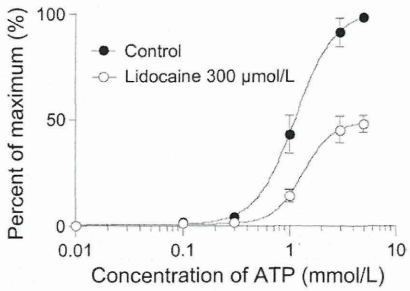


**Figure 4.** Characterization of the inhibitory effects of lidocaine on adenosine triphosphate (ATP)-induced currents in oocytes expressing P2X7 receptor. We measured the currents induced by 10 μmol/L to 5 mmol/L ATP in the absence and presence of 300 μmol/L lidocaine. Lidocaine did not change the half maximal effective concentration ($EC_{50}$) value and the the slope variable (hill coefficient) (control, 1.2 ± 0.1 mmol/L and 3.7 ± 0.9; lidocaine, 1.4 ± 0.2 mmol/L and 4.8 ± 1.1), however, it significantly reduced the maximal response ($E_{max}$) value to 49 ± 4% of control ($P$ < 0.0001; 99% confidence interval, −65.7 to −35.1). Data are presented as means ± SEM ($n$ = 6). Hill coefficient, $EC_{50}$, and $E_{max}$ values were calculated using GraphPad Prism. Data were statistically evaluated by unpaired $t$ test (two-tailed) using the same software.

## Characterization of the Inhibitory Effects of Lidocaine on the P2X7 Receptor

To determine whether lidocaine competes with ATP for the P2X7 receptor, we next investigated the effects of 300 μmol/L lidocaine on the ATP concentration–response curve. ATP-induced currents at concentrations of 10 μmol/L to 5 mmol/L were measured in the absence and presence of lidocaine. As shown in Figure 4, lidocaine-mediated inhibition was not overcome by increasing the ATP concentration. In addition, lidocaine significantly reduced the $E_{max}$ value (maximal response) of the ATP concentration–response curve to 49% ± 4% ($P$ < 0.0001; 99% confidence interval [CI], −65.7 to −35.1). The $EC_{50}$ and slope variables in the absence and presence of lidocaine were 1.2 ± 0.1 mmol/L and 3.7 ± 0.9, and 1.4 ± 0.2 mmol/L and 4.8 ± 1.1, respectively. Thus, lidocaine significantly suppressed $E_{max}$ without significantly changing $EC_{50}$ ($P$ = 0.385; 99% CI, −0.279 to 0.665) and slope variables ($P$ = 0.483; 99% CI, −2.165 to 4.268), suggesting noncompetitive inhibition.

## Effects of Charged and Uncharged Local Anesthetics on the P2X7 Receptor

We next assessed the effects of QX-314, which is a permanently charged and non–membrane-permeable lidocaine analog, on the P2X7 receptor. When applied extracellularly, QX-314 inhibited ATP-induced currents in a concentration-dependent manner; the $IC_{50}$ value and slope variable were 500 ± 75 μmol/L and 0.36 ± 0.03, respectively (Fig. 5B, Table 1). Moreover, intracellular QX-314 injection also significantly suppressed ATP-activated currents to 51 ± 9% of the control, whereas injected 150 mmol/L KCl had no effect (112 ± 11% of the control) (Fig. 5C). These results suggest that QX-314 can act on the P2X7 receptor both extracellularly and intracellularly, and the charged lidocaine can suppress the function of P2X7. Therefore, we investigated whether charge is required for the inhibitory actions of local anesthetics on the P2X7 receptor by measuring the effects of benzocaine, a local anesthetic that is almost completely uncharged and highly membrane permeable. Benzocaine suppressed the response to ATP in a concentration-dependent manner with the $IC_{50}$ value and the slope variable of 1.6 ± 0.04 mmol/L and 0.88 ± 0.07, respectively (Fig. 5B, Table 1). These data suggest that both charged and uncharged local anesthetics can suppress P2X7 receptor function although it remains unclear whether benzocaine acts intracellularly or extracellularly.

## Analyzing the Use Dependency of Lidocaine-Mediated Inhibition of the P2X7 Receptor

We assessed whether the effects of lidocaine on the P2X7 receptor were use-dependent because local anesthetics exhibit use-dependent block of voltage-gated sodium channel function, ATP was applied to oocytes at 5-minute intervals in the absence or continuous presence of 100 μmol/L lidocaine for 30 minutes (Fig. 6A). In the continuous presence of lidocaine, the response to the second application of ATP (after 5 minutes) was significantly reduced to 66 ± 3% of the current induced by the first application (0 minute), and the inhibitory effect of the seventh application (30 minutes) was similar to that of the second application (5 minutes) (Fig. 6, B and C). Therefore, these results revealed the effectiveness of lidocaine as it reached steady state in the second application of ATP (5 minutes), suggesting that lidocaine-mediated inhibition of the P2X7 receptor is use-dependent. More potent inhibition might be observed in the second application compared with the first because lidocaine would be able to access its site of

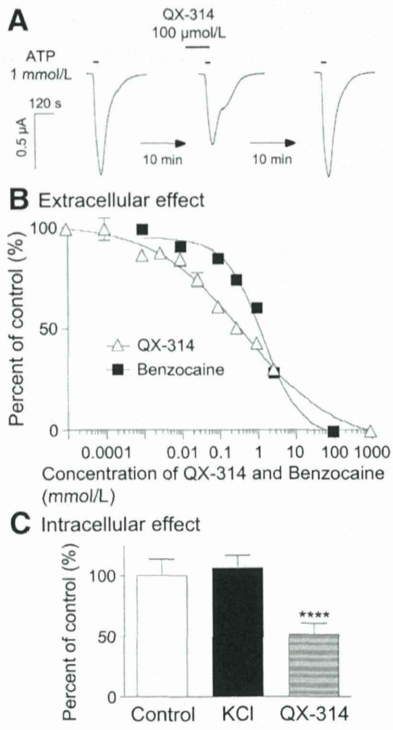


**Figure 5.** Effects of *N*-(2,6-dimethylphenylcarbamoylmethyl) triethylammonium bromide (QX-314) and benzocaine on adenosine triphosphate (ATP)-induced currents in oocytes expressing P2X7 receptor. A, Representative traces from oocytes expressing P2X7 receptors in both the absence and presence of extracellular 100 μmol/L QX-314. ATP was applied for 20 seconds with or without pretreatment for 2 minutes with 100 μmol/L QX-314. The currents of QX-314-treated oocytes were recorded 10 minutes after recording the control currents, and the washout currents were obtained 10 minutes after QX-314 treatment. B, Concentration–response curves for extracellular QX-314 and benzocaine-mediated inhibition on ATP-induced currents in oocytes expressing P2X7 receptors. The peak current amplitude in the presence of local anesthetics was normalized to that of the control, and the effects are expressed as percentages of the control. QX-314 and benzocaine reduced ATP-induced currents in a concentration-dependent manner with the half maximal inhibitory concentration ($IC_{50}$) values and slope variables of 500 ± 75 μmol/L and 0.36 ± 0.03, and 1.6 ± 0.04 mmol/L and 0.88 ± 0.07, respectively. C, Effects of intracellular QX-314 on ATP-induced currents in oocytes expressing P2X7 receptor. ATP-induced currents were recorded 10 minutes after intracellular injection of 5 mmol/L QX-314 diluted by 150 mmol/L potassium chloride (KCl), resulting in intracellular concentration of 500 μmol/L and intracellular injection of 150 mmol/L KCl as control cells. Intracellular injection of QX-314 significantly suppressed ATP-induced currents to 51 ± 9% of control although that of 150 mmol/L KCl did not reduce those currents. Data are presented as means ± SEM ($n$ = 6). Hill coefficient and $IC_{50}$ values were calculated using GraphPad Prism. ****$P$ < 0.0001 compared with the control (one-way analysis of variance followed by Dunnett post hoc test).

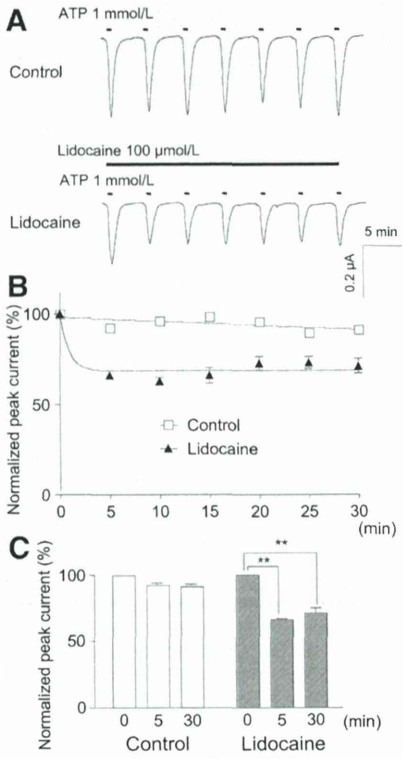


**Figure 6.** Analyses of use dependency of lidocaine block on P2X7 receptor. Repetitive application of 1 mmol/L adenosine triphosphate (ATP; 20 seconds) at 5-minute intervals was done in the absence or continuous presence of 100 μmol/L lidocaine for 30 minutes. A, Representative traces induced by repetitive ATP application for 30 minutes from oocytes expressing P2X7 receptors in the absence or continuous presence of 100 μmol/L lidocaine. All typical traces were obtained from the same oocyte. B, Peak currents were normalized to the first pulse and plotted against the pulse number. C, Comparison of the normalized currents between the first (0 minute), second (5 minutes), and seventh (30 minutes) applications. In the continuous presence of lidocaine, the responses to the second application of ATP (5 minutes) were significantly reduced to 66% ± 3% of currents induced by the first application of ATP (0 minute). However, inhibitory effect at seventh application (30 minutes) was statistically the same as that at second application (5 minutes). Data are expressed as means ± SEM ($n$ = 6). **$P$ < 0.01 compared with the control (one-way analysis of variance followed by Tukey post hoc test).

action sufficiently during a 5-minute treatment, whereas the first application in which lidocaine and ATP were simultaneously applied (no lidocaine pretreatment) would not be sufficient for lidocaine to access its site of action.

### Effects of Selective P2X7 Receptor Antagonists on the Inhibitory Actions of Lidocaine

We next investigated the action of lidocaine in the absence and presence of selective antagonists of the P2X7 receptor, BBG, or AZ11645373 to determine whether these compounds modulate the inhibitory actions of lidocaine on the P2X7 receptor. Oocytes were pretreated with 1 μmol/L BBG or 300 nmol/L AZ11645373 2 minutes before coapplication of 10 μmol/L to 3 mmol/L lidocaine for 2 minutes (Fig. 7, A and B). Figure 7, C and D, shows normalized inhibition curves for lidocaine in the absence and presence of preapplied and coapplied BBG or AZ11645373. The $IC_{50}$ values and slope variables of the lidocaine concentration–response curves with BBG or AZ11645373 were 315 ± 56 μmol/L and 0.92 ± 0.14, or 258 ± 52 μmol/L and 0.93 ± 0.18, respectively. This suggests that neither BBG nor AZ11645373 modulated the effects of lidocaine, which exhibited an $IC_{50}$ value and slope variable of 282 ± 45 μmol/L and 0.72 ± 0.07, respectively ($P$ = 0.658 and 0.237, or $P$ = 0.736 and 0.309, respectively, for the $IC_{50}$ values and the slope variables of the lidocaine concentration–response curves with BBG or AZ11645373, 99% CI, −132.7 to 198.7 and −0.161 to 0.561, or −134.6 to 182.6 and

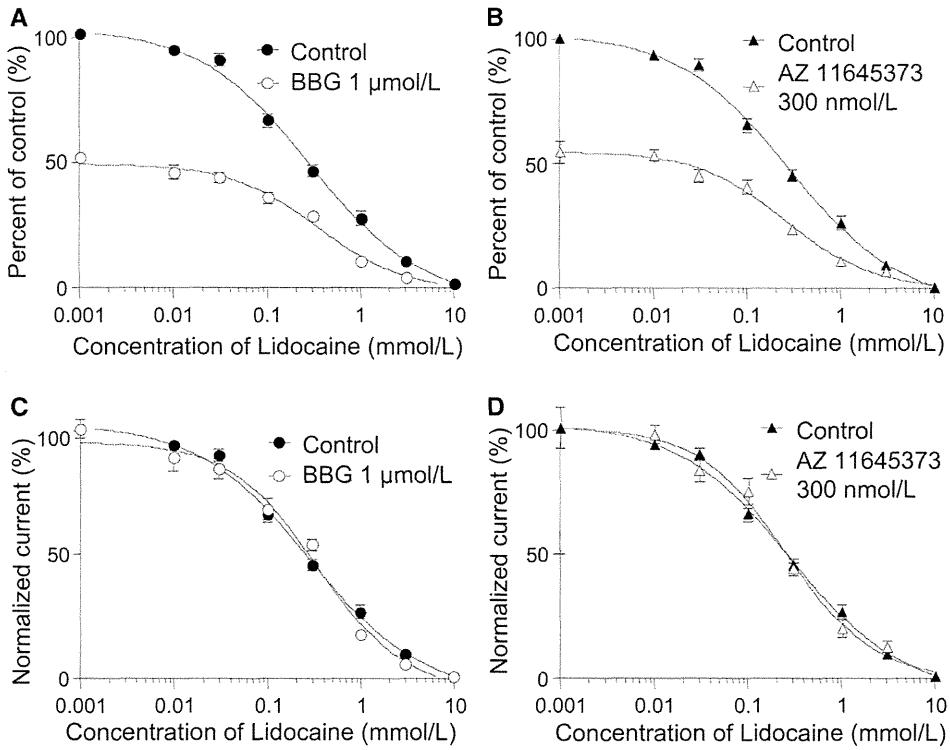


**Figure 7.** Consentration-response curves for lidocaine inhibition in the absence or presence of pre-treatment for 4-min by 1 μmol/L BBG (A) or 300 nmol/L AZ11645373 (B). BBG or AZ11645373 were pre-applied for 2 min prior to co-application with lidocaine (10 μmol/L–3 mmol/L) for 2 min. Normalized inhibition curves for lidocaine in the absence and presence of BBG (C) or AZ11645373 (D) were obtained from (A) and (B). Both BBG and AZ11645373 did not affect inhibitory effect of lidocaine on P2X7 receptor; the IC50 values and slope variables were 282 ± 45 μmol/L and 0.72 ± 0.07, 315 ± 56 μmol/L and 0.92 ± 0.14, and 258 ± 52 μmol/L and 0.93 ± 0.18, in control, BBG, and AZ11645373 pre-treatment, respectively. Data are expressed as means ± SEM ($n$ = 6). Hill coefficient, IC50 values were calculated using GraphPad Prism. ATP = adenosine triphosphate; BBG = Brilliant Blue G; IC50 = half maximal inhibitory concentration.

−0.235 to 0.655). Overall, these data suggest that lidocaine interacts with a different site on the P2X7 receptor from the sites of action of either BBG or AZ11645373, which are noncompetitive antagonists of the P2X7 receptor.

## DISCUSSION

In the present study, we demonstrated that lidocaine selectively inhibited ATP-induced inward currents of the P2X7 receptor in a concentration-dependent manner. To our knowledge, this study is the first direct evidence that lidocaine suppresses the P2X7 receptor. Substantial pain relief is achieved at plasma concentrations of 2 to 5 μg/mL (7–20 μmol/L) by continuous infusion of lidocaine in cancer patients with neuropathic pain.[33] In the present study, the $IC_{50}$ value of lidocaine-mediated P2X7 inhibition was 282 ± 45 μmol/L. Lidocaine tended to suppress ATP-induced currents at concentrations ≥10 μmol/L, and these inhibitory effects were significant at concentrations ≥30 μmol/L ($P$ < 0.001). Although it is not proven whether a small inhibitory effect (7% inhibition) of lidocaine at 10 μmol/L produces pain relief in systemic administration, lidocaine might suppress P2X7 function at least when it is administered locally such as epidural administration because P2X7 receptors are expressed on glial cells in spinal neurons.

Lidocaine had little effect on the P2X3 and P2X4 receptors, but selectively suppressed the function of P2X7 receptors. The P2X7 receptor is structurally distinct from other P2X receptors; it also has different gating properties although these are only poorly understood.[34] The P2X7 receptor is permeable not only to small cations (sodium, potassium, and calcium) as similar to other subunits but also to larger cations such as *N*-methyl-D-glucamine and nanometer-sized dyes,[35] probably through the progressive dilation of the pore or the opening of a distinct accessory channel.[36] Therefore, it is of great interest to explore how lidocaine inhibits the function of the P2X7 receptor alone. Lidocaine would not affect three ATP-binding sites that are shown to exist in the extracellular region of P2X receptors[37] because lidocaine-mediated inhibition was found to be noncompetitive in this study. Moreover, lidocaine-mediated inhibition of the P2X7 receptor was use-dependent. Taken together, these findings suggest that lidocaine exerts its effects by affecting the site in the ion channel pore. By contrast, the inhibitory effects of QX-314 and benzocaine suggest that both charged and uncharged local anesthetics can also suppress P2X7 function. Moreover, intracellular injection of the charged local anesthetic QX-314 also inhibited P2X7 function. These results suggest that lidocaine acts on both intracellular and extracellular sites of the P2X7 receptor and that both charged and uncharged lidocaine could modulate this receptor.

We demonstrated that other local anesthetics including mepivacaine, ropivacaine, and bupivacaine have only limited effects on P2X7 receptor function. All these three

compounds, but not lidocaine, contain a piperidine ring (Fig. 8), to which is attached a carbon chain of different lengths; one carbon in mepivacaine, three in ropivacaine, and four in bupivacaine. Therefore, it is possible that the piperidine ring is an obstacle to the action of local anesthetics because the inhibitory potency of mepivacaine was one-twentieth of that of lidocaine. Moreover, longer carbon chains connected to the piperidine ring may further hinder potential effects because both ropivacaine and bupivacaine had little effect on the P2X7 receptor. Therefore, it is possible that lidocaine suppresses P2X7 receptor function by acting on a binding site in the ion channel. Recent X-ray crystal structural analyses of P2X4 receptor in zebrafish revealed that P2X receptors exhibit homotrimeric architecture and that each subunit consists of a large hydrophilic extracellular domain and a transmembrane domain composed of two α-helices, which resemble the shape of a dolphin.[38,39] These analyses also revealed that binding of ATP to the ATP-binding pocket within an intersubunit cleft rotates each subunit, resulting in promotion of the ion channel pore opening. P2X3, P2X4, and P2X7 are 40% to 50% identical in amino acid sequence, and each subunit of the P2X7 receptor has a longer amino acid sequence (595) than P2X3 (397) and P2X4 (388) because only P2X7 has a long intracellular C-terminus. Therefore, lidocaine might bind to the binding pocket for local anesthetics that exists mainly in the P2X7 receptor because of the differences of amino acid sequence to prevent the subunits from rotating that leads to channel opening.

Many P2X7 receptor antagonists have been reported in addition to antagonists of other P2X receptor subunits although they are not used clinically.[34] Some antagonists, including pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid and periodate-oxidized ATP, were demonstrated to interact at the ATP-binding sites, showing that they were competitive antagonists.[40] By contrast, many of them inhibit P2X7-mediated responses by acting in a noncompetitive manner. We examined whether BBG and AZ11645373 interact with the inhibitory action of lidocaine on P2X7 receptors because these two compounds are selective P2X7 inhibitors and act in a similar noncompetitive manner to lidocaine. BBG was shown to produce a noncompetitive inhibition of rat P2X7 receptors more potently than human P2X7 receptors; $IC_{50}$ values were 10 and 200 nmol/L, respectively,[41] whereas

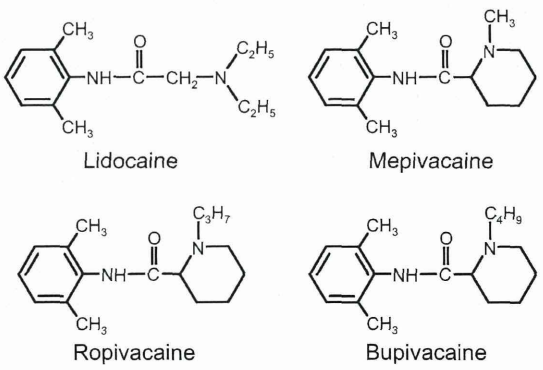


**Figure 8.** Structures of lidocaine, mepivacaine, ropivacaine, and bupivacaine.

it was reported that AZ11645373 is a highly selective and potent antagonist at human, but not at rat, P2X7 receptors.[42] Our data suggested that lidocaine acts on a different site of the P2X7 receptor than both BBG and AZ11645373.

The systemic administration of lidocaine has been considered to reduce neuropathic pain via suppression of ectopic activity by inhibiting abnormally expressed voltage-gated sodium channels.[5] One study demonstrated that the incidence of C-fibers with ongoing activity was significantly reduced by systemic administration of lidocaine resulting in low plasma concentrations in a chronic inflammatory model.[43] However, it was also shown that this effect was only partially correlated with chronic pain relief compared with the complete inhibition of spontaneous discharge in C-fibers, suggesting that there may be mechanisms other than blocking sodium channel activity. Moreover, many reports demonstrate that lidocaine also affects other pain signaling pathways.[6] It has been demonstrated that intravenous lidocaine infusion increased acetylcholine concentrations in cerebrospinal fluid, which exacerbated inhibitory descending pain pathways resulting in analgesia.[44] Lidocaine has also been shown to produce central inhibitory effects via spinal strychnine-sensitive glycine receptors[45] and to stimulate the release of endogenous opioids to promote its analgesic effect.[46] Moreover, lidocaine has been reported to reduce the postsynaptic depolarization mediated by *N*-methyl-D-aspartate and neurokinin receptors,[47] and a study in a recombinant model demonstrated that local anesthetics, including lidocaine, directly inhibited the activation of human *N*-methyl-D-aspartate receptors in a concentration-dependent manner.[48]

The P2X7 receptor is expressed predominantly on immune cells and has been shown to play an important role in the inflammatory response by demonstrating that activation of the P2X7 receptor leads to maturation and release of interleukin (IL)-1β and initiation of a cytokine cascade.[49,50] Several reports also suggest a role for the P2X7 receptor in pain modulation because systemic administration of selective antagonists of P2X7 receptors produced antinociceptive effects,[51] and hypersensitivity was not observed in P2X7-knockout mice[52] in neuropathic and inflammatory pain models. In addition, some reports suggest that lidocaine exerts anti-inflammatory effects by suppressing cytokine-induced injury[53] or attenuating the production of proinflammatory cytokines including tumor necrosis factor-α, IL-1β, and IL-6 induced by extracellular ATP in microglia.[54] Taken together, these data indicate that P2X7 receptor antagonists might be beneficial for the treatment of neuropathic and inflammatory pain. Therefore, suppression of the P2X7 receptor might be a mechanism underlying the anti-inflammatory effects and chronic pain relief affected by lidocaine. Although our present results suggest that the P2X3 and P2X4 receptors are not involved in the mechanism of lidocaine-induced pain relief, further experiments are needed to investigate the effects on other subunits including P2X2/3 or P2Y12, which are also related to chronic pain. Although ropivacaine and bupivacaine can relieve neuropathic pain effectively by epidural administration, P2X7 signaling would not be involved in their mechanism underlying pain relief.

In conclusion, lidocaine selectively inhibited ATP-induced currents of P2X7 receptors expressed in *Xenopus*

oocytes at clinically relevant concentrations when it was administered locally at least. The effect of lidocaine on P2X7 receptors was likely a result of noncompetitive inhibition at both extracellular and intracellular sites in the ion channel pore. To our knowledge, these results are the first evidence showing novel lidocaine-mediated effects on P2X receptors in a recombinant experimental system and might become the key to elucidate the mechanisms of pain relief by lidocaine. However, further studies are needed to clarify the relevance of P2X7 receptor inhibition of the analgesic effects of lidocaine.

## DISCLOSURES

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**Attestation:** Dan Okura approved the final manuscript, and attests to the integrity of the original data and the analysis reported in this manuscript.
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Original article

# Development of an experimentally useful model of acute myocardial infarction: 2/3 nephrectomized triple nitric oxide synthases-deficient mouse

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ABSTRACT

We investigated the effect of subtotal nephrectomy on the incidence of acute myocardial infarction (AMI) in mice deficient in all three nitric oxide synthases (NOSs). Two-thirds nephrectomy (NX) was performed on male triple NOSs$^{-/-}$ mice. The 2/3NX caused sudden cardiac death due to AMI in the triple NOSs$^{-/-}$ mice as early as 4 months after the surgery. The 2/3NX triple NOSs$^{-/-}$ mice exhibited electrocardiographic ST-segment elevation, reduced heart rate variability, echocardiographic regional wall motion abnormality, and accelerated coronary arteriosclerotic lesion formation. Cardiovascular risk factors (hypertension, hypercholesterolemia, and hyperglycemia), an increased number of circulating bone marrow-derived vascular smooth muscle cell (VSMC) progenitor cells (a pro-arteriosclerotic factor), and cardiac up-regulation of stromal cell-derived factor (SDF)-1α (a chemotactic factor of the progenitor cells) were noted in the 2/3NX triple NOSs$^{-/-}$ mice and were associated with significant increases in plasma angiotensin II levels (a marker of renin–angiotensin system activation) and urinary 8-isoprostane levels (a marker of oxidative stress). Importantly, combined treatment with a clinical dosage of an angiotensin II type 1 receptor blocker, irbesartan, and a calcium channel antagonist, amlodipine, markedly prevented coronary arteriosclerotic lesion formation and the incidence of AMI and improved the prognosis of those mice, along with ameliorating all those pro-arteriosclerotic parameters. The 2/3NX triple NOSs$^{-/-}$ mouse is a new experimentally useful model of AMI. Renin–angiotensin system activation, oxidative stress, cardiovascular risk factors, and SDF-1α-induced recruitment of bone marrow-derived VSMC progenitor cells appear to be involved in the pathogenesis of AMI in this model.



## 1. Introduction

Acute myocardial infarction is a disorder in which cardiac myocytes undergo necrosis as a consequence of interrupted coronary blood flow [1]. Acute myocardial infarction is a major cause of morbidity and mortality worldwide, with more than 7 million people in the world suffering from acute myocardial infarction each year [1]. Over the past two decades, the in-hospital mortality rate after admission for acute myocardial infarction has substantially declined to less than 10%, owing to

*Abbreviations:* ACE, angiotensin-converting enzyme; ADMA, asymmetric dimethylarginine; AMI, acute myocardial infarction; APC, activated protein C; apo E, apolipoprotein E; $AT_1$, angiotensin II type 1; CKD, chronic kidney disease; ECG, electrocardiography; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL, high-density lipoprotein; mAb, monoclonal antibody; NO, nitric oxide; NOS, NO synthase; NX, nephrectomy; Sca-1$^+$, stem cell antigen-1$^+$; SDF-1α, stromal cell-derived factor-1α; VSMC, vascular smooth muscle cell; WHHL, Watanabe heritable hyperlipidemic; WT, wild-type.

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