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Local Anesthetics Inhibit the G Protein-Mediated Modulation of K^+ and Ca^{++} Currents in Anterior Pituitary Cells

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ABSTRACT

The effects of local anesthetics (LAs) on G protein-mediated responses of voltage-dependent K^+ (I_K) and Ca^{++} currents in rat anterior pituitary tumor (GH_3) cells were analyzed by using a whole-cell voltage clamp. Extracellular lidocaine inhibited I_K with an IC_{50} of 1.9 mM, comparable to 2.6 mM for I_{Ba} but 10 times higher than the IC_{50} for I_{Na} (0.17 mM). Low concentrations of lidocaine (30–100 μM), which had no direct effect on basal I_K, attenuated both the stimulatory and inhibitory modulation of K^+ channels by thyrotropin-releasing hormone (TRH). Both modulations had an IC_{50} ~40 μM independent of [TRH]. Intracellular QX314 (100 μM), a quaternary, charged form of lidocaine, also significantly attenuated the TRH effects; however, external QX314 and the neutral LA benzocaine (100 μM) did not. Lidocaine (≤100 μM) inhibited the TRH-induced increase in $[Ca^{++}]$ but failed to block either the GTP-γ-S-induced increase in I_K, the activation of I_K by directly elevated $[Ca^{++}]$ (ca. 3×10^{-7} M), or the phorbol-12,13-dibutyrate-induced inhibition of Ca^{++}-activated I_K. Agonist binding assays revealed that none of the these LAs affected TRH receptor binding. Similar to its effect on TRH modulation of I_K, lidocaine (100 μM) attenuated the inhibition of Ca^{++} channels in GH_3 cells by somatostatin (1 μM). These results suggest that lidocaine's action occurs between agonist binding and G protein activation. Such inhibition of G protein pathways may be an important component of the general action of LAs acting at spinal sites, or for i.v. therapeutics or during cardiotoxic episodes.

At clinical analgesic concentrations for nerve block (final tissue concentrations of 1–4 mM), local anesthetics (LAs) show a variety of effects, including modulation of different types of ion channels and catalytic enzymes (cf. review by Butterworth and Strichartz, 1990). In addition to a primary role in impulse blockade through the inhibition of Na^+ channels, LAs inhibit voltage-gated Ca^{++} and K^+ channels (Bacaner et al., 1986; Josephson, 1988; Castle, 1990), Ca^{++}-activated K^+ channels (Benham et al., 1985; Oda et al., 1992), ATP-sensitive K^+ channels (Yoneda et al., 1993), and various ligand-gated channels or other cell membrane functions (see Butterworth and Strichartz, 1990). The modulation of ion channels is mediated by a broad variety of membrane-intrinsic proteins (such as protein kinases) as well as by members of the superfamily of receptors that are coupled to G proteins (Li et al., 1995). vs may also regulate the intracellular processes of Ca^{++} movement. For example, it has been shown that lidocaine inhibits spontaneous Ca^{++} release from sarcoplasmic reticulum (Volpe et al., 1983) and Ca^{++} release from platelet membrane vesicles induced by inositol triphosphate (Seiler et al., 1987). In GH_4 cells, lidocaine (≥1 mM) and procaine (≥2.5 mM) inhibited the increase in $[Ca^{++}]_i$ induced by 100 nM TRH or 30 mM K^+ (Wang et al., 1990). Therefore, the mechanisms of LAs during spinal anesthesia and i.v. analgesia, as well as on vascular tone, may well extend beyond Na^+ channel blockade, and the range of actions of submillimolar concentrations of LAs that occur in these situations deserves investigation.

In a separate study (Z. Xiong, P. Albert, and G. R. Strichartz, submitted), we report that in GH_3 cells thyrotropin-releasing hormone (TRH) evokes biphasic changes of voltage-gated potassium currents, namely, a transient increase followed by a sustained inhibition. Both phases are shown to be mediated by G proteins: the pertussin toxin (PTX)-insensitive $G_{q/11}$ proteins for the stimulatory phase, and the PTX-sensitive G_i proteins for the inhibitory phase. In the present study, these clonal pituitary GH_3 cells have been used to examine possible mechanisms of low concentrations of LAs. We report a novel finding, that lidocaine at low concentrations blocks the G protein-mediated responses.

Materials and Methods

Cell Culture. Rat pituitary GH_3 cells were purchased from the American Type Culture Collection and cultured as described previ-

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ABBREVIATIONS: LAs, local anesthetics; TRH, thyrotropin-releasing hormone; PDBu, phorbol-12,13-dibutyrate; PKC, protein kinase C; SMS, somatostatin; MEM, minimum essential medium; HP, holding potential; TP, testing potential; PEI, polyethylenimine; PTX, pertussin toxin; TEA, tetrathylammonium.

ously (Dubinsky and Oxford, 1984). Briefly, cells were grown on 12-mm glass coverslips placed in 35-mm plastic tissue culture dishes. The culture medium was 89% Dulbecco's modified Eagle's medium (catalog no. 11965; GIBCO Laboratories, Life Technologies, Inc., Grand Island, NY) supplemented with 1% penicillin/streptomycin (GIBCO catalog no. 15140) and 10% fetal bovine serum (catalog no. A-1111; Hyclone Laboratories, Logan, UT). Cells were kept in a humidified incubator at 37°C in a 5% CO_2/95% air-gas mixture.

Electrophysiology. The methods used to record the whole-cell membrane currents were similar to those described previously (Hamill et al., 1981). The coverslip (with cells attached) was placed in a small chamber (0.6 ml) positioned on the stage of a differential interference inverted microscope (IMT-2; Olympus Co., Tokyo, Japan). Only round cells with total cell capacitance of 15 to 50 pF (mostly 20–30 pF, ca. 13–20 μm in diameter) were used. The chamber was then perfused continuously during experimentation at a flow rate of 2 ml/min. Drugs were flushed onto the voltage-clamped cell through 0.2-mm (i.d.) glass tubes placed 0.5 mm away from the cell. In some experiments, the intracellular perfusion was conducted using an intracellular perfusion technique in which a special electrode holder (30 degree model) (E.W. Wright, Guilford, CT) and a quartz tubing (0.1 mm i.d.) (Adams & List Associates, LTD, Westbury, NY) were used. All experiments were performed at room temperature (22–24°C).

Whole-cell voltage clamp was performed with patch electrodes made from borosilicate glass capillary tubing (World Precision Instruments, Inc., Sarasota, FL) (with resistance of 0.8–3 MΩ for standard voltage-clamp experiments or 0.5–0.8 MΩ for intracellular perfusion experiments) using a patch-clamp amplifier (Axopatch-200; Axon Instruments, Inc., Foster City, CA). After formation of a giga-seal (>10 GΩ), the patch membrane was ruptured by negative pressure (10–20 mm H_2O). Series resistance (R_s, 1.5–4 MΩ) was partly (70%) compensated electrically. The current signal from the amplifier was filtered at 2 kHz (4-pole Bessel filter). Leak and residual capacitive currents were subtracted using the P/4 procedure.

Data Expression. All data were recorded and analyzed using pClamp software (Axon Instruments). The data are expressed as means ± S.E.M.. Student's *t* test was used to examine the statistical significance between paired groups. Any difference with a *P* value of less than .05 was considered to be statistically significant. All current levels, unless otherwise specified, were measured at the end of a 300-ms depolarizing test pulse.

Solutions. To evoke I_K, the pipette was filled with a high K^+ solution of the following composition: 137 mM KCl, 5 mM $MgCl_2$, 5 mM Na_2ATP, 0.2 MM EGTA, and 10 mM HEPES. The bath was superfused with a physiological salt solution with the following composition: 137 mM NaCl, 6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES. To measure Ca^{++} channel current (I_{Ba}), the pipette was filled with a high Cs^+ solution of the following composition: 110 mM CsCl, 20 mM tetraethylammonium (TEA)-Cl, 5 mM $MgCl_2$, 5 mM Na_2ATP, 0.2 mM EGTA, and 10 mM HEPES. The cells were superfused with a 10 mM Ba^{++} solution with the following composition: 130 mM TEA-Cl, 10 mM $BaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES. The Ba^{++} ion was the dominant charge carrier. Tetrodotoxin (0.3 μM) was added in the solution to eliminate the Na^+ channel current (I_{Na}). The pH was adjusted to 7.2 for the pipette solution and 7.35 for the bath solutions by using Tris.

Drugs. The drugs used were TRH, lidocaine, somatostatin (SMS; Sigma, St. Louis, MO) benzocaine, QX314 (kind gift from Astra Pharmaceutical Products, Inc., Worcester, MA), phorbol-12,13-dibutyrate (PDBu), and tetrodotoxin (Calbiochem Corp., San Diego, CA).

TRH Receptor Binding Assay. GH_3 cells were grown in 12-well plates for 3 days to about 70% confluence. Each well was washed once with 1 ml of serum-free minimum essential medium (MEM) and then filled with 1 ml of serum-free MEM containing LAs or vehicle alone. Cells were incubated therein at 37°C for 30 min and then chilled on ice. Subsequent binding assays were performed in 0.75 ml of serum-free MEM containing 20 nM [^{3}H]MeTRH (NEN-DuPont, Boston, MA) and LAs or vehicle alone at 4°C to measure cell surface TRH receptor number. Nonspecific binding was determined in the presence of 1 mM unlabeled MeTRH. After a 3-h incubation at 4°C, each well was rinsed three times with ice-cold phosphate-buffered saline. Cells were then solubilized in 0.75 ml of 0.1 N NaOH, and bound TRH was counted in scintillation fluid (Aquasol 2; NEN-DuPont).

$[Ca^{++}]_i$ Measurement. Intracellular Ca^{++} ($[Ca^{++}]_i$) was determined by dual excitation microfluorometry by using the Ca^{++}-sensitive fluorescent dye, Fura-2, as described in GH_3 cells by Bukusoglu and Sarlak (1996). Excitation wavelengths were 340 and 380 nm, and the emitted light was passed through a 510-nm interference

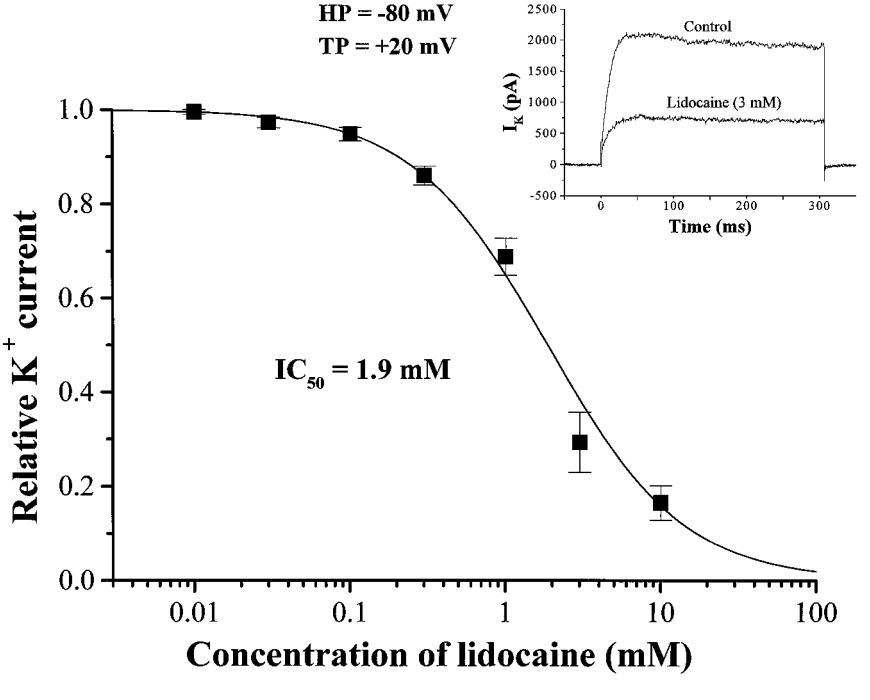

Fig. 1. Concentration-dependent inhibition by lidocaine of voltage-dependent potassium current (I_K) in GH_3 cells. The bath contained a physiological salt solution, and the pipette contained a high K^+ solution with low concentration (0.2 mM) of EGTA. The HP was −80 mV, and the testing potential (TP) was +20 mV. As shown, the concentration for half-inhibition is 1.9 mM and no significant inhibition occurred at concentrations below 100 μM. The curve was fitted by Hill equation with a Hill coefficient of 0.99. An example of the I_K inhibition by 3 mM lidocaine (5 min after addition) is given (upper right).

filter to a photomultiplier tube. The concentration of $[Ca^{++}]_i$ was calculated according to the formula of Grynkiewcz et al. (1985):

$$[Ca^{++}]_i = K_d \times b \times (R - R_{min})/(R_{max} - R) \quad (1)$$

in which $r = F_{340}/F_{380}$, $R_{max} = R$ in digitonin-treated cells in 2.5 mM Ca^{++}, and $R_{min} = R$ in the same cells after the subsequent addition of 10 mM EGTA to the bath. K_d was taken as 224×10^{-9} M (Grynkiewcz et al., 1985). The b (Sf2/Sb2) was determined in separate experiments. The background fluorescence was measured in the presence of digitonin and subtracted from all measurements before calculation.

Results

Effect of LAs on K^+ *Currents in Clonal Anterior Pituitary (GH_3)* Cells. Depolarization of GH_3 cells to potentials more positive than −50 mV from a holding potential (HP) of −80 mV produces outward K^+ currents (Dubinsky and Oxford, 1984; Mollard et al., 1988; Z. Xiong, Albert, and G. R. Strichartz, submitted). Application of lidocaine extracellularly suppressed I_K in a concentration-dependent manner, with an IC_{50} of 1.9 mM (Fig. 1); inhibition was fully and rapidly reversible. This lidocaine concentration is near that for inhibition of $I_{Ca.}$ (IC_{50} = 2.6 mM; Xiong and Strichartz, 1995). At a concentration of 100 μM or less, lidocaine exerted little effect on I_K (Figs. 1 and 2Ba); this justified the use of these low concentrations in the following experiments to study their effects on TRH-induced changes in I_K.

Blockade by LAs of TRH-Induced Changes of K^+ Currents in GH_3 Cells. Extracellular application of TRH (0.03–1 μM) produces a transient increase in I_K (peaking at ca. 0.1 min), followed by a sustained inhibition (Fig. 2Aa) (Dubinsky and Oxford, 1984; Z. Xiong, Albert, and G. R. Strichartz, submitted). For example, TRH at 1 μM increased I_K (after 300 ms of depolarization) to 2.17 ± 0.24 (n = 7) times control at 1 min, and decreased I_K to 0.65 ± 0.03 times control at 3 min (Fig. 2Ab). These phases are due to different, independently acting G proteins (Z. Xiong, Albert, and G. R. Strichartz, submitted). The stimulatory phase is driven directly by a transient elevation of intracellular Ca^{++} released from internal stores by IP_3, whose production is catalyzed by

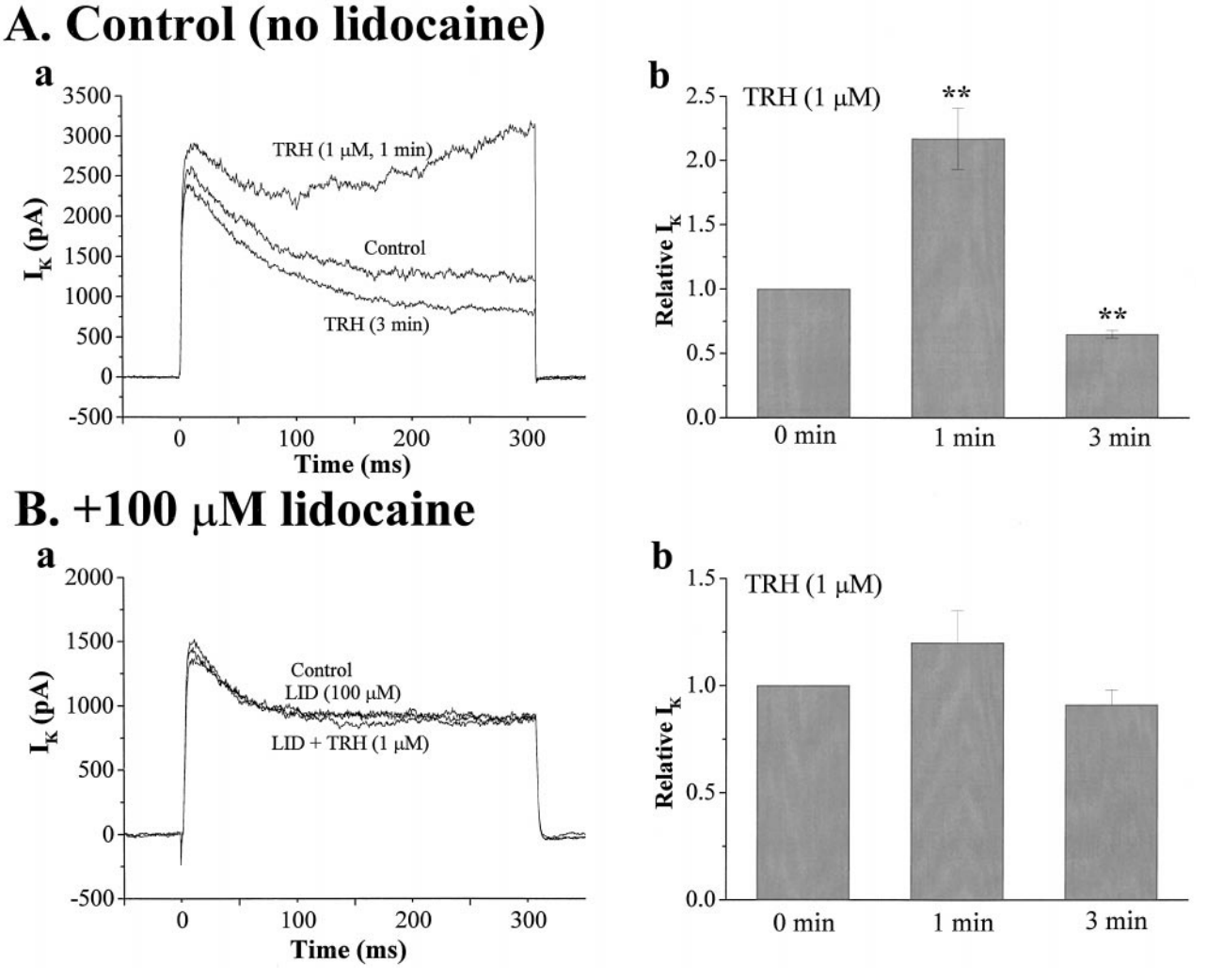

Fig. 2. Effect of TRH on I_K in the absence (A) and presence (B) of lidocaine in GH_3 cells. The experimental conditions were the same as in Fig. 1. Aa, an example of TRH-induced dual effect on I_K. Ab, average data of TRH-induced responses. The columns and the bars denote mean ± S.E. (n = 7). "0 min" represents before TRH; "1 min" and "3 min" represent 1 min and 3 min after TRH, respectively. ** $P < .01$ compared with control (0 min). Ba, lidocaine blockade of TRH-induced changes in I_K. As shown, lidocaine itself had no effect on I_K, but prevented TRH-induced effect at both 1 min [lido + TRH (1 μM)] and 3 min (trace not shown). Lidocaine was administered extracellularly for 5 min before TRH. Ba, average data of TRH-induced responses. The columns and bars denote mean ± S.E. (n = 7). "0 min" represents before TRH; "1 min" and "3 min" represent 1 min and 3 min after TRH, respectively. Both $P > .05$ compared with control (0 min).

phospholipase C and probably activated by $G_{q/11}$ G proteins. The inhibitory phase has an unknown mechanism but is independent of intracellular Ca^{++} and is abolished by G_i protein family knockout and by pertussis toxin pretreatment.

To investigate the role of LAs on these G protein-mediated responses, lidocaine, its quaternary derivative (permanently charged) QX314, and benzocaine (permanently neutral) were applied before and during TRH addition. As shown in Fig. 2B, lidocaine at 100 μM largely prevented the modulatory effects of TRH (1 μM) on I_K. At 1 min, TRH only increased I_K to 1.20 ± 0.15 (n = 7) times control at 1 min and decreased I_K to 0.91 ± 0.07 times control at 3 min (Fig. 2Bb). Both values are significantly smaller ($P < .01$) than those obtained without lidocaine treatment (see Fig. 2Ab). Pretreatment of cells with lidocaine for 5 to 10 min was necessary to get the maximal inhibitory effect. The TRH-induced response was unaffected when both lidocaine (100 μM) and TRH (1 μM) were applied simultaneously (n = 3; data not shown).

Anomalously, the TRH effect was occasionally was not blocked by 100 μM lidocaine, even when pretreated for 10 min (n = 3). The reason for this heterogenous response is not clear, but it may be related to the cell age and intrinsic conditions that regulate the coupling of the intracellular process.

The concentration-dependence for lidocaine's inhibition of TRH-induced changes in I_K is summarized in Fig. 3. Inhibition was significant at lidocaine concentrations of 30 to 100 μM, and almost equal for both stimulatory and inhibitory actions. For example, 100 μM lidocaine reduced the TRH (1 μM)-induced stimulatory effect by 83 ± 6.5% (Fig. 3A) and the inhibitory effect by 74 ± 8.6% (n = 7) (Fig. 3B). Interestingly, there was no difference in lidocaine's potency for inhibition at the different TRH concentrations. The IC_{50} values of lidocaine for blocking the stimulation of I_K induced by 0.1, 0.5, and 1 μM TRH (EC_{50} ~67 nM) were 35.3, 32.2, and 37.0 μM, respectively. Similarly, the IC_{50} values for blocking the inhibition of I_K induced by these same TRH concentrations (EC_{50} ~190 nM) were 37.9, 36.0, and 40.7 μM, respectively.

A related LA, QX314, which is a permanently charged, quaternary form of lidocaine (Frazier et al., 1970; Strichartz, 1973), also could suppress the actions of TRH. Extracellular application of 100 μM QX314 for 5 min (Fig. 4A) neither inhibited I_K (control, 1635 ± 369 pA; QX 314, 1525 ± 278 pA, n = 9, $P > .5$) nor prevented the TRH (0.2 μM)-induced biphasic effect. Potassium current was increased by TRH to 1.89 ± 0.23 of the control at 1 min and declined to 0.85 ± 0.03 at 3 min (n = 9), virtually the same as in the control (1.90 ± 0.09 at 1 min and 0.80 ± 0.07 at 3 min). But when QX314 was applied to the intracellular side, with an internal perfusion technique described previously (Xiong et al., 1991), the response to TRH was strongly attenuated (Fig. 4B). After application of TRH (0.2 μM) to the bath, I_K increased only to 1.23 ± 0.09 of control at 1 min and declined to 0.93 ± 0.02 at 3 min, n = 5. These values are significantly smaller than those of the respective TRH responses in the absence of intracellular QX314, 26% of the stimulatory phase, and 35% of the inhibitory phase ($P < .05$ for both). In contrast, at 100 μM, intracellular QX314 directly inhibited I_K by only 9% (control: 2976 ± 848 pA, QX314: 2706 ± 672 pA, n = 5, $P > .2$).

A third LA, benzocaine (a permanently neutral form), applied extracellularly at 100 μM, had no direct effect either on I_K or on the TRH-induced responses (n = 3) (data not shown). Benzocaine is quite hydrophobic (Strichartz et al., 1992) and will rapidly equilibrate across the cell membrane, reaching an intracellular concentration equal to its bathing solution value in less than 1 min (Hille, 1977). Intracellular perfusion of benzocaine is therefore not required for it to reach any cytoplasmic target.

Effect of Lidocaine on SMS-Induced Inhibition of I_{Ba}. To examine the effect of lidocaine on a different G protein (G_o)-mediated ligand response, SMS was used to modulate Ca^{++} channels. In the control condition, SMS (1 μM) inhibited Ba^{++} current (I_{Ba}) (Fig. 5A). The peak effect occurred at about 3 min after application with an average inhibition of 15.0 ± 2.3% (n = 7; $P < .01$). In contrast, when the cells were pretreated for 5 to 10 min with 100 μM lido-

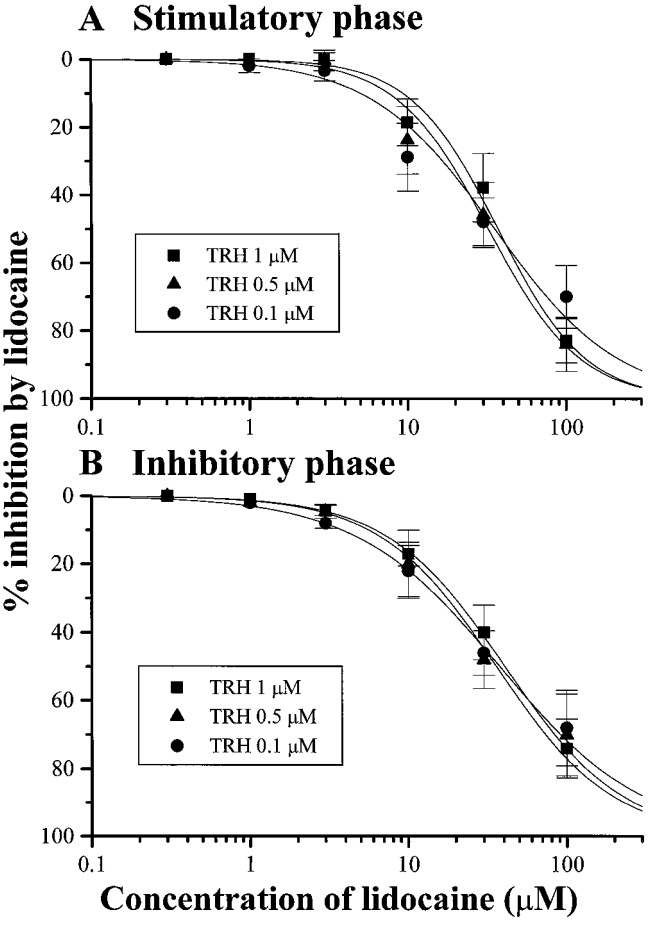

Fig. 3. Inhibitory potency of lidocaine on TRH-induced changes in I_K of both stimulatory and inhibitory phases. Cells were pre-exposed for 5 min to various concentrations of lidocaine to assess their inhibition of the TRH (0.1, 0.5, and 1 μM)-induced effects. The data were obtained from I_K evoked by a voltage step to +20 mV (TP) from a −80 mV (HP). The experimental solutions were the same as in Fig. 1. A, percent inhibition by lidocaine of stimulatory phase of TRH action. B, percent inhibition by lidocaine of inhibitory phase of TRH action. All data are expressed in means ± S.E. from 3 to 10 cells. Curves are fits of the equation (logistic)

$$(A1 - A2)/\{1 + ([LID]/IC_{50})^p\} + A2 \quad (1)$$

where A1 denotes the maximum amplitude, A2 denotes the minimum amplitude, and p denotes the slope. The IC_{50} of [LID] was 35.3, 32.2, and 37.0 μM, respectively, for blocking 0.1, 0.5, and 1 μM TRH-induced stimulation. For blocking these concentrations of TRH-induced inhibition, the IC_{50} was 37.9, 36.0, and 40.7 μM, respectively.

caine, at which concentration I_{Ba} was directly reduced by ~6%, the inhibitory effect of SMS (1 μM) was almost abolished (Fig. 5B). The averaged value is 2.3 ± 1.0% (n = 5), only 15% of the change of current in the control group. Thus, lidocaine inhibited equally the effects of SMS on Ca^{++} channels and the two effects of TRH on K^+ channels, even though three different G proteins are involved in these separate actions.

Lack of Effect of LAs on TRH Receptor Binding. To test the possibility that LAs are modulating TRH binding to its receptor on the cell membrane, a radioactive TRH binding assay was performed. Two concentrations of a TRH agonist ([^{3}H]MeTRH; 10 and 20 nM) near the K_D were chosen so that LA-induced changes in receptor density or in agonist affinity would appear as changes in the specific ligand binding. Neither lidocaine nor QX314 at concentrations of 100 μM affected the specific binding of 20 nM [^{3}H]MeTRH. The value of specific binding (cpm) was 2357.7 ± 92.9 for control, 2252.9 ± 53.8 for lidocaine, and 2218.0 ± 107.2 for QX314 (n = 3, P > .5). Similar findings were observed at 10 nM [^{3}H]MeTRH, wherein 100 μM lidocaine increased the saturable binding to 109% of control, 100 μM QX314 increased it to 115%, and 100 μM benzocaine increased it to 106%. None of these values differs significantly from those of the control.

Effect of Lidocaine on GTP-γ-S-Activated I_K. Intracellular perfusion of GH_3 cells with GTP-γ-S increases I_K to a new steady-state level in 2 to 5 min (Z. Xiong, P. Albert, and G. R. Strichartz, submitted). To characterize the relationship between lidocaine actions and G proteins in this study, the cells were preincubated with lidocaine before GTP-γ-S perfusion. In the absence of lidocaine, GTP-γ-S (100 μM) increased I_K by 2.92 ± 0.75-fold (n = 7), whereas in the presence of 100 μM lidocaine, GTP-γ-S (100 μM) increased I_K by 1.91 ± 0.45-fold (n = 6). The difference between these two groups is not statistically significant (P > .2), and the overall inhibition by lidocaine is far less than the 80% effect on TRH's action (cf. Fig. 3).

Effect of LAs on $[Ca^{++}]_i$ Level. Because TRH transiently increased I_K by augmentation of intracellular Ca^{++} released from intracellular stores (Z. Xiong, P. Albert, and G. R. Strichartz, submitted), here we have tested whether lidocaine interferes with the TRH-triggered release of intracellular Ca^{++}. Representative tracings are shown in Fig. 6. In the pilot experiments, the EC_{50} for the TRH-induced in-

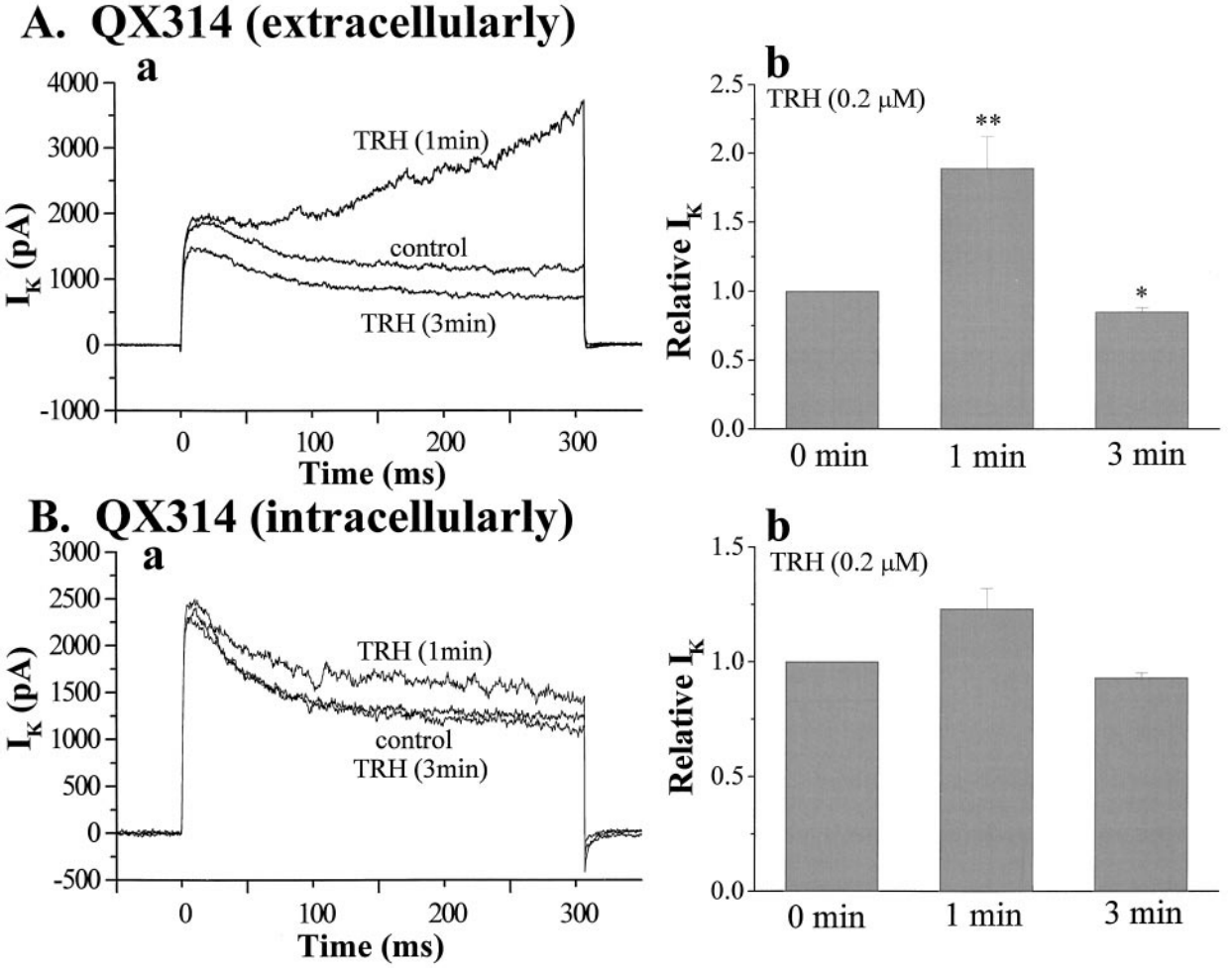

Fig. 4. Effect of QX314 (100 μM) on TRH-induced changes in I_K. The HP was −80 mV, and the TP was +20 mV. The experimental solutions were the same as in Fig. 1. Aa, when QX314 was applied extracellularly, no effect on TRH-induced effect was observed. Ab, averaged results of this experiment show that the transient stimulatory phase at 1 min after TRH and the steady inhibitory phase at 3 min in the presence of 100 μM QX314 are still significantly different from the baseline currents (P < .01 and < 0.05, respectively). Ba, when QX314 was applied intracellularly, a substantial reduction of the TRH-induced effect (for both stimulatory and inhibitory) was observed, as shown by the average results (Bb) where no significant change from baseline currents was detected at 1 or 3 min.

crease in $[Ca^{++}]_i$ was approximately 2.5 nM. A concentration of 3 nM TRH was therefore used in this experiment. Figure 6A shows the reaction of two repeated applications of TRH on $[Ca^{++}]_i$. In this assay, TRH evoked an initial transient increase in $[Ca^{++}]_i$, which was sometimes followed by a delayed, small, sustained component. With an interval of 10 min between two sequential TRH applications, no desensitization or enhancement of the TRH response was observed. The average increase in $[Ca^{++}]_i$ by 3 nM TRH was 231 ± 39 nM for the first application and 254 ± 40 nM for the second application ($n = 3$).

In cells pretreated with lidocaine (100 μM) for 5 to 10 min, the TRH-induced increase of $[Ca^{++}]_i$ was attenuated. This effect of lidocaine, however, was highly variable. It seemed largely dependent on conditions such as number of cell culture passages and the age and confluence of the cells. For example, lidocaine strongly inhibited the TRH-induced $[Ca^{++}]_i$ peak transient by 61.0 ± 8.1% ($n = 5$) in one cell culture dish (Fig. 6B). In a separate cell culture dish, however, lidocaine inhibited the peak $[Ca^{++}]_i$ increase by only 10.8 ± 4.7% ($n = 4$) (Fig. 6C). In these coverslips the confluence of GH_3 cells was similar. The average inhibition from 15 experiments is 35.1 ± 7.4%. Lidocaine (100 μM) itself had no effect on basal $[Ca^{++}]_i$. Similarly, at this concentration, lidocaine had no effect on the 30 mM K^+-induced increase in $[Ca^{++}]_i$ in GH_3 cells (data not shown), consistent with its IC_{50} of 2.6 mM for Ca^{++} channel currents (Xiong and Strichartz, 1995).

Effect of LA on Activation of I_K *by Increased Intracellular* Ca^{++}. To examine whether LAs (e.g., lidocaine) directly inhibit Ca^{++}-activated I_K, an internal perfusion solution containing elevated free Ca^{++} (3×10^{-7} M) was used. This concentration of Ca^{++} is approximately the value of peak Ca^{++} induced by TRH as detected in the intracellular Ca^{++} measurement experiments (see Fig. 6) and is high enough to activate Ca^{++}-dependent K^+ channels in clonal anterior pituitary cells (Wong et al., 1982). Potassium currents were markedly enhanced by the elevated intracellular $[Ca^{++}]$ (Fig. 7A); the average increase was 6.60 ± 0.82 times the control ($n = 5$). Lidocaine (100 μM), applied extracellularly, did not block the Ca^{++} activation of I_K (Fig. 7B), which still had an average value of 5.73 ± 1.10 times the control ($n = 6$) ($P > .5$).

Effect of Lidocaine on PDBu-Induced Inhibition of I_K. The phorbol ester PDBu, an activator of protein kinase C (PKC), significantly inhibits TRH's stimulation of Ca^{++}-ac-

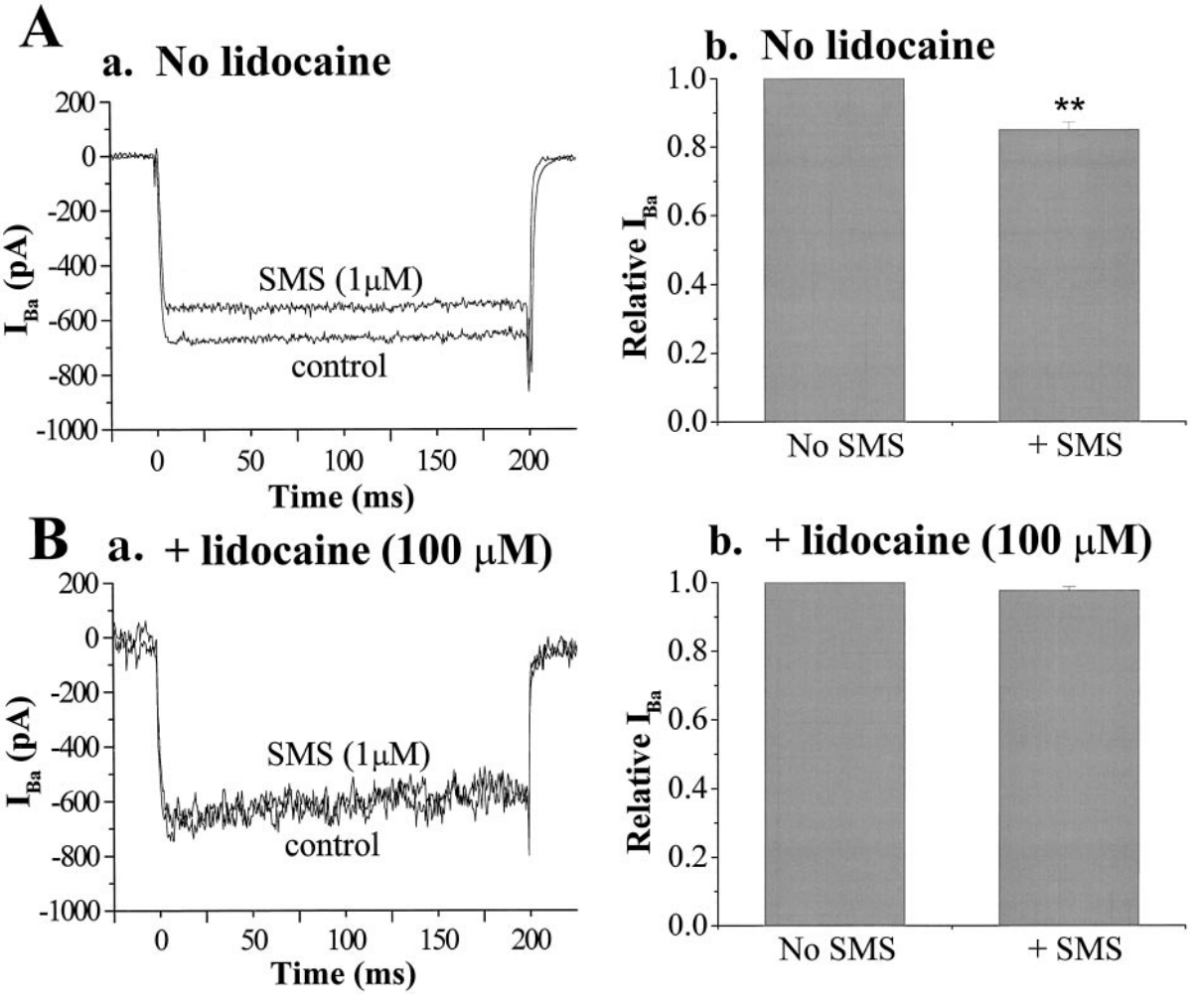

Fig. 5. Effect of SMS (1 μM) on I_{Ba} in the absence (A) and presence (B) of lidocaine (100 μM). Lidocaine was present in the bath for 8 min before application of SMS. The HP was −80 mV, and the TP was −10 mV. The intracellular pipette contained a high Cs^+ solution, and the bath contained 10 mM Ba^{++} solution. Aa, treatment with SMS reduces I_{Ba} in this lidocaine-free example, and Ab in the average of seven cells (**$P < .01$), but in cells pretreated with lidocaine (Ba) there is no significant change in I_{Ba} induced by SMS ($n = 5$, $P > .5$ for the average (Bb).

tivated I_K but does not affect the inhibitory phase of TRH action (Z. Xiong, P. Albert, and G. R. Strichartz, submitted). The I_K in cells not stimulated by TRH is inhibited by PDBu by ca. 10%, the same degree to which this I_K was reduced by charybdotoxin, a specific blocker of Ca^{++}-activated K^+ channels. To test lidocaine's influence on the actions of PKC, the effect of PDBu on Ca^{++}-activated I_K was compared in the absence and presence of lidocaine (100 μM). Without lidocaine, PDBu (0.1 μM) inhibited I_K by 10.1 $\pm$ 1.4% (n = 5). In the presence of 100 μM lidocaine preapplied for 5 to 10 min, this value was virtually unchanged (9.8 $\pm$ 1.7%, n = 4). ($P >$ .5 for the two groups). Phosphorylation of the channel, or of some modulatory proteins, by activated PKC is not affected by lidocaine at a concentration that blocks 70 to 80% of TRH's modulatory effects.

Discussion

In the present experiments, lidocaine, at concentrations that did not block K^+ channels directly, significantly inhibited the TRH-induced changes in I_K, with an IC_{50} of approximately 40 μM (Fig. 3). The tertiary amine LA (lidocaine), but not the neutral drug benzocaine nor the permanently charged, membrane-impermeant cationic LA (QX314) applied extracellularly, inhibited the TRH-induced changes in I_K. Intracellular QX314 was a potent inhibitor, however, at 100 μM; it reduced the stimulatory and inhibitory phases by amounts approximately equal to those affected by 100 μM extracellular lidocaine. If we assume that extracellular 100 μM lidocaine equilibrates with the cytoplasmic compartment after several minutes [which, given its known membrane permeability (Bernards and Hill, 1992), is likely], then the concentration of charged protonated lidocaine inside the cell at pH 7.2, one pH unit below the pK_a (Strichartz et al., 1992), equals 91 μM. Within the variance of the measured inhibitions, therefore, lidocaine and QX314 are equipotent. The selective activity of intracellular QX compounds resembles closely the inhibition of Na^+ channels seen with lidocaine and QX314 (Frazier et al., 1970; Strichartz, 1973) and suggests that the cationic species of the drug acting at the cytoplasmic surface produces the observed inhibition. The necessity for lidocaine to permeate the plasma membrane in order to exert its actions on the cytoplasmic surface provides a possible explanation for the requirement of a 5-min preincubation period to establish inhibition of TRH's actions. In separate experiments measuring the rate of inhibition of I_{Na} by extracellularly applied LAs, a delay of less than 10 s separated drug perfusion and effect (data not shown), demonstrating that perfusion dead time or drug diffusion within the bath is not the rate-limiting step for lidocaine's actions on the TRH response.

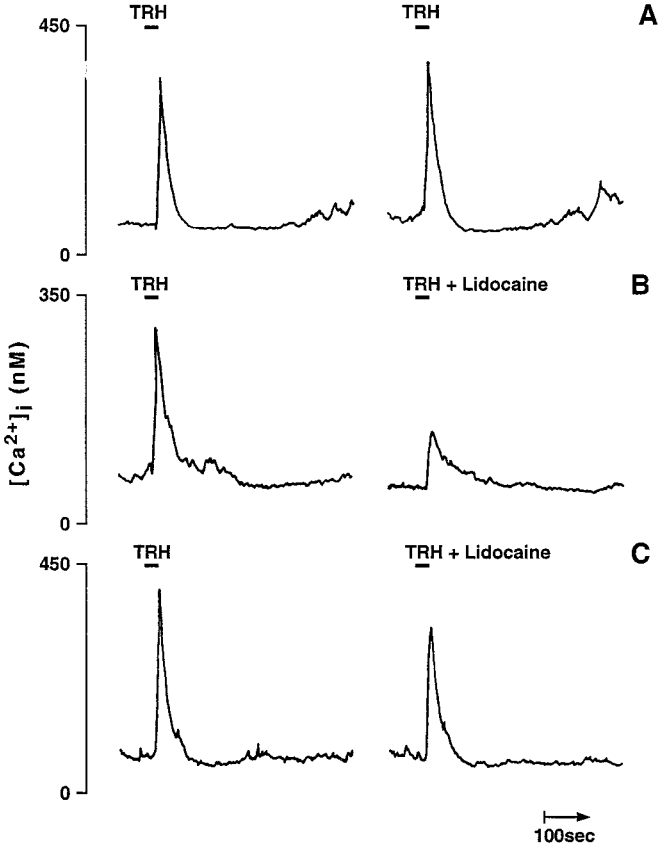

Fig. 6. Effect of lidocaine on TRH-induced changes in intracellular Ca^{++} ($[Ca^{++}]_i$) levels. A, transient increase in $[Ca^{++}]_i$ upon a repetitive application of TRH (3 nM) in a control coverslip. Left, first application; right, second application. The interval between them was 10 min. B, TRH (3 nM)-induced $[Ca^{++}]_i$ change in the absence (left) and presence (for 10 min, right) of lidocaine (100 μM). As shown, $[Ca^{++}]_i$ was significantly suppressed by lidocaine. C, TRH (3 nM)-induced $[Ca^{++}]_i$ change in the absence (left) and presence (for 10 min, right) of lidocaine (100 μM) in another coverslip from a different passage of the cell culture. As shown, $[Ca^{++}]_i$ was only slightly inhibited by lidocaine.

We found that TRH evoked a biphasic effect on I_K: a transient increase followed by a sustained decrease. The stimulatory action is the result of augmentation of Ca^{++}-activated K^+ currents, whereas the inhibitory action may be a more direct inhibition on K^+ channels. Both phases of TRH response appear to be mediated by G proteins (Z. Xiong, P. Albert, and G. R. Strichartz, submitted). Thus, the possible mechanisms of LAs on the TRH-induced response include 1) blocking TRH receptors on the cell membrane; 2) uncoupling G proteins, which are activated by TRH receptors; 3) blocking intracellular Ca^{++} release, thereby reducing the Ca^{++}-activated I_K; 4) directly interfering with Ca^{++}'s activation of K^+ channels; and 5) some unknown intermediary cascades.

Because TRH binding is unaltered by lidocaine (as well as by QX 314) at concentrations that almost totally block the modulation of I_K, the possibility of TRH receptor blockade can be ruled out. In addition, lidocaine (100 μM) had no effect on the increase of I_K induced by directly elevated intracellular Ca^{++}. Thus, an action at the Ca^{++}-modulating site as well as a direct inhibition by lidocaine of Ca^{++}-activated I_K can also be eliminated. Similarly, direct PKC involvement is also unlikely because lidocaine failed to prevent PDBu-induced inhibition on I_K.

LAs Prevent Increase in $[Ca^{++}]_i$. If LAs decrease the release of Ca^{++} from intracellular stores, they will inhibit the TRH-induced increase in I_K. Several lines of evidence indicate that LAs can alter intracellular Ca^{++} levels, although the mechanisms may be different. For example, in a murine cell line expressing Substance P (NK1) receptors, bupivacaine, lidocaine, tetracaine, and benzocaine blocked both receptor binding and the $[Ca^{++}]_i$ increase (by Substance P), although relatively high concentrations were needed (IC_{50} $\geq$500 μM) (Li et al., 1995). In GH_4 cells, lidocaine ($\geq$1 mM) and procaine ($\geq$2.5 mM) inhibited the increment in $[Ca^{++}]_i$ induced by both TRH (100 nM) and 30 mM K^+ (Wang

et al., 1990). Because of the high concentrations of LAs required (IC_{50} about 3 mM), this result could be due to a direct inhibition of agonist binding or of membrane ion channels (i.e., Ca^{++} and K^+ channels), or to some other nonspecific mechanisms. On the other hand, low concentrations of lidocaine (IC_{50} = 50 μM) inhibit the 1,4,5-trisphosphate (IP_3)-induced Ca^{++} release from isolated platelet membrane vesicles (Seiler et al., 1987). In the present experiments, low concentrations of lidocaine effectively blocked the TRH- but not the K^+-induced increase in $[Ca^{++}]_i$ (Fig. 7), suggesting that IP_3-induced Ca^{++} release from SR was decreased, by either a direct inhibition of SR Ca^{++} releasing channels (Volpe et al., 1983), or by a less direct pathway, such as uncoupling of G protein receptors (see below), thereby decreasing IP_3 generation.

The effect of LAs on TRH-induced changes in $[Ca^{++}]_i$ was, however, highly variable. In some coverslips, lidocaine exhibited strong inhibition of the TRH-induced response, but in others it had only a minor effect (see Fig. 6, B and C). Consistent with this finding, the TRH effect on I_K was occasionally not blocked by 100 μM lidocaine, even when pretreated for 10 min (n = 3). The reason for this heterogenous response is not clear, but it may be related to the cell age and intrinsic conditions that regulate the coupling of intracellular process. A heterogenous intracellular Ca^{++} response to the coapplication of pregnenolone sulfate with Bay K 8644 was also found in GH_3 cells (Bukusoglu and Sarlak, 1996), indicating that the coupling of G protein-dependent membrane steroid receptors to voltage-gated Ca^{++} channels is also variable.

Do LAs Uncouple Membrane G Proteins? As mentioned above, the TRH-induced changes in I_K are mediated by G proteins (such as G_q/G_{11}, and G_i families) (Z. Xiong, P. Albert, and G. R. Strichartz, submitted). Because both lidocaine and intracellular QX314 prevent these effects of TRH, as does GDP-β-S (a G protein antagonist), and because GTP-γ-S itself mimics the TRH stimulatory response, which is not blocked by lidocaine, it is conceivable that these LAs *uncouple* G proteins from the receptors on the cell membrane. This is consistent with the observation that a low concentration (100 μM) of lidocaine effectively blocked somatostatin-induced inhibition on I_{Ba} (Fig. 5B), which is a G_o protein-mediated response (Kleuss, 1995; Degtiar et al., 1997). Thus, we hypothesize that some LAs can nonselectively uncouple G protein signal transduction. Results from a study of nonhydrolyzable guanine nucleotide analogs stimulating cyclase activity regulated by *beta* adrenergic receptors suggest an indirect, modulatory effect on cyclase rather than a direct action by LAs. That is, preincubation of membrane-bound receptors with isoproterenol and Gpp(NH)p led to a stimulation of adenylyl cyclase activity that could be inhibited by tetracaine if it was present in the preincubation period but not if it was added after cyclase activation (Voeikov and Lefkowitz, 1977). Other experiments showed that the extent of inhibition of adenylyl cyclase by tetracaine in the preincubation mixture depended on the concentration of guanine nucleotide and that the guanine nucleotide-induced dissociation of agonist was accelerated by tetracaine. The results thus indicate an action of LAs on the G protein-binding/activating site of the *beta* adrenergic receptor molecule (Voeikov and Lefkowitz, 1977). Evidence from other nonneural cells also suggests that LAs act on G proteins. For example, in rat peritoneal mast cells it has been shown that the polycations polyethylenimine (PEI) (such as PEI_6 and PEI_{12}) induced a histamine release that is mediated by G proteins. Lidocaine (1 mM) was able to inhibit the histamine release induced by PEI, an inhibition that was partly reversed by the addition of polycation (Suzuki-Nishimura et al., 1995). The observation in the present experiments of virtually identical potencies for lidocaine's inhibition of both

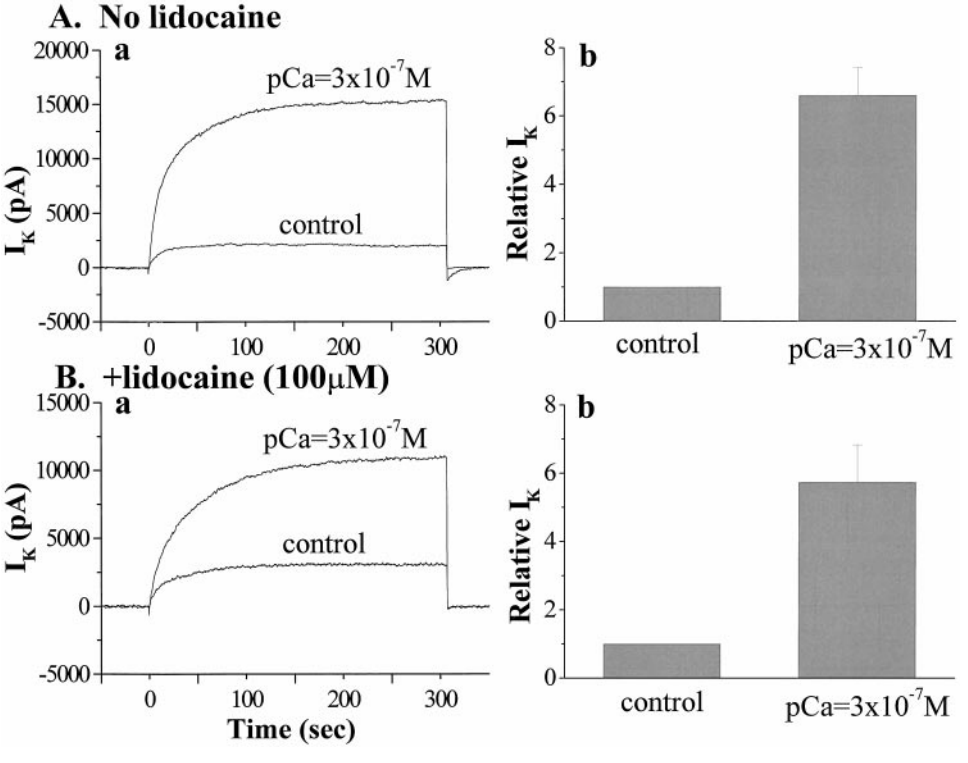

Fig. 7. Effect of intracellular Ca^{++} ion (3×10^{-7} M) on I_K. The HP was −80 mV and the TP was +20 mV. A, in the absence of lidocaine, I_K was markedly increased after 1.5 min intracellular perfusion of Ca^{++} as shown by the single example (Aa) and in the graph of the average effect (Ab, n = 5). B, in the presence of 100 μM lidocaine in the bath solution for 10 min, I_K was still significantly increased after 1.5 min intracellular perfusion of Ca^{++} shown by the example of Ba and in the average results (Bb, n = 6).

phases of TRH-induced changes in I_K is consistent with a common site at the TRH receptor, since there are different second messenger pathways for the increase and decrease of I_K, unlikely to be equally susceptible to both LAs. However, because the results in the present study were obtained by observing the effect of LAs on I_K, and the intracellular factors affecting this current are certainly broad, other mechanisms such as protein phosphorylation cannot be ruled out.

In summary, lidocaine at low concentrations can prevent an agonist-induced, G protein-coupled response. This novel finding may enable us to better understand the broad functions of LAs that occur during clinical use.

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References

Bacaner MB, Clay JR, Shrier A and Brochu RM (1986) Potassium channel blockade: A mechanism for suppressing ventricular fibrillation. *Proc Natl Acad Sci USA* **83:**2223–2227.

Benham CD, Bolton TB, Lang RJ and Takewaki T (1985) The mechanism of action of Ba^{2+} and TEA on single Ca^{2+}-activated K^+-channels in arterial and intestinal smooth muscle cell membranes. *Pflügers Arch* **403:**120–127.

Bernards CM and Hill HF (1992) Physical and chemical properties of drug molecules governing their diffusion through the spinal meninges. *Anesthesiology* **77:**750.

Bukusoglu C and Sarlak F (1996) Pregnenolone sulfate increases intracellular Ca^{2+} levels in a pituitary cell line. *Eur J Pharmacol* **298:**79–85.

Butterworth JF and Strichartz GR (1990) Molecular mechanisms of local anesthesia: A review. *Anesthesiol* **72:**711–734.

Castle NA (1990) Bupivacaine inhibits the transient outward K^+ current but not the inward rectifier in rat ventricular myocytes. *J Pharmacol Exp Ther* **255:**1038–1046.

Degtiar VE, Harhammer R and Nurnberg B (1997) Receptors couple to L-type calcium channels via distinct G_o proteins in rat neuroendocrine cell lines. *J Physiol (Lond)* **502:**2:321–333.

Dubinsky JM and Oxford GS (1984) Ionic currents in two strains of rat anterior pituitary tumor cells. *J Gen Physiol* **83:**309–339.

Frazier DT, Narahashi T and Yamada M (1970) The site of action and active form of local anesthetics. II. Experiments with quaternary compounds. *J Pharmacol Exp Thera* **171:**45–51.

Grynkiewcz G, Poenie M and Tsien RY (1985) A new generation of $[Ca^{2+}]$ indicators with greatly improved fluorescence properties. *J Biol Chem* **260:**3440–3450.

Hamill OP, Marty A, Neher E, Sakmann B and Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch* **391:**85–100.

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

Josephson IR (1988) Lidocaine blocks Na, Ca, and K currents of chick ventricular myocytes. *J Mol Cellu Cardiol* **20:**593–604.

Kleuss C (1995) Somatostatin modulates voltage-dependent Ca^{2+} channels in GH_3 cells via a specific $G_{(o)}$ splice variant. *Ciba Found Symp* **190:**171–182.

Li YM, Wingrove DE, Too HP, Marnerakis M, Stimson ER, Strichartz GR and Maggio JE (1995) Local anesthetics inhibit substance P binding and evoked increases in intracellular Ca^{2+}. *Anesthesiol* **82:**166–173.

Mollard P, Vacher P, Dufy B and Barker JL (1988) Somatostatin blocks Ca^{2+} action potential activity in prolactin-secreting pituitary tumor cells through coordinate actions on K^+ and Ca^{2+} conductances. *Endocrinology* **123:**721–732.

Oda M, Yoshida A and Ikemoto Y (1992) Blockade by local anesthetics of the single Ca^{2+}-activated K^+ channel in rat hippocampal neurons. *Br J Pharmacol* **105:**63–70.

Seiler SM, Arnold AJ and Stanton HC (1987) Inhibitors of inositol trisphosphate-induced Ca^{2+} release from isolated platelet membrane vesicles. *Biochem Pharmacol* **36:**3331–3337.

Strichartz GR (1973) The inhibition of sodium currents in myelinated nerve by quternary derivatives of lidocaine. *J Gen Physiol* **62:**37–57.

Strichartz GR, Sanchez V, Arthur R, Chafetz R and Martin D (1992) Fundamental properties of local anesthetics. II. Measured octanol: buffer partition coefficients and pK_a values of clinically used drugs. *Anesth Anal* **71:**158–170.

Suzuki-Nishimura T, Oku N, Nango M and Uchida MK (1995) PEI_6, a new basic secretagogue in rat peritoneal mast cells: Characteristics of polyethylenimine PEI_6 resemble those of compound 48/80. *Gen Pharmacol* **26:**1171–1178.

Voeikov VV and Lefkowitz RJ (1977) Effect of local anesthetics on guanyl nucleotide modulation of the catecholamine-sensitive adenylate cyclase system and β-adrenergic receptors. *Biochem Biophys Acta* **629:**266–281.

Volpe P, Palade P, Costello B, Mitchell RD and Fleischer S (1983) Spontaneous calcium release from sarcoplasmic reticulum: Effect of local anesthetics. *J Biol Chem* **258:**12434–12442.

Wang X, Sato N and Greer MA (1990) Lidocaine and procaine inhibit the increase in cytosol Ca^{2+} induced by thyrotropin-releasing hormone or K^+ depolarization in GH_4C_1 cells. *Mol Cellu Endocrinol* **74:**185–190.

Wong BS, Lecar H and Adler M (1982) Single calcium-dependent potassium channels in clonal anterior pituitary cells. *Biophys J* **39:**313–317.

Xiong ZL, Kitamura K and Kuriyama H (1991) ATP activates cationic currents and modulates the calcium current through GTP-binding protein in rabbit portal vein. *J Physiol* **440:**143–165.

Xiong ZL and Strichartz GR (1995) Inhibition by local anesthetics of calcium channels in anterior pituitary cells. *Biophys J* **68:**209.

Yoneda I, Sakuta H, Okamoto K and Watanabe Y (1993) Effects of local anesthetics and related drugs on endogenous glibenclamide-sensitive K^+ channels in *Xenopus* oocytes. *Eur J Pharmacol* **247:**267–272.

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