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Local Anesthetic Inhibition of G Protein-Coupled Receptor Signaling by Interference with $G\alpha_q$ Protein Function

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ABSTRACT

Although local anesthetics are considered primarily Na^+ channel blockers, previous studies suggest a common intracellular site of action on different G protein-coupled receptors. In the present study, we characterized this site for the LPA, m1 muscarinic, and trypsin receptor. *Xenopus laevis* oocytes expressing endogenous LPA and trypsin or recombinant m1 receptors were two-electrode voltage clamped. We studied LPA inhibition in the presence of ropivacaine stereoisomers to determine whether LA act on a protein site. Ropivacaine inhibited LPA signaling in a stereoselective and noncompetitive manner, suggesting a protein interaction. Antisense injection was used to characterize G protein α-subunits involved in mediation of LPA, m1, trypsin, and angiotensin$_{1A}$ receptor signaling. Lidocaine and its analog QX314 were injected into oocytes expressing these receptors to examine a potential role for specific G protein α-subunits as targets for LA. $G\alpha_q$ was shown to be among the primary G protein subunits mediating the LPA, m1, and trypsin receptor signaling, all of which were inhibited to a similar degree by intracellular injected QX314 (424×10^{-6} M). Since the angiotensin$_{1A}$ receptor, previously shown not to be affected by LA, was found not to signal via $G\alpha_q$, but via $G\alpha_o$ and $G\alpha_{14}$, the intracellular effect of LA most likely takes place at the $G\alpha_q$-subunit.

Although blockade of Na^+ channels is the primary mode of local anesthetic action, in the past decade alternative actions of LA have increasingly become a topic of investigation and may lead to new clinical uses for these compounds (such as anti-inflammatory indications). We have previously reported inhibitory effects of LA on several G protein-coupled receptors, such as thromboxane A_2 (Hoenemann et al., 1998), m1 muscarinic acetylcholine (Hollmann et al., 1999), and in particular LPA receptors (Nietgen et al., 1997). LPA is likely to be a wound-healing mediator, making such investigations particularly relevant, because LA are used frequently for injection around surgical wounds. We have shown that lidocaine or bupivacaine inhibits LPA, but not angiotensin, signaling. QX314 (a permanently charged and hence membrane-impermeant lidocaine analog) inhibited LPA signaling only when injected intracellularly, and benzocaine (permanently uncharged LA) inhibited with a similar half-maximal inhibitory concentration (IC_{50}). Combined administration of both compounds exerted superadditive effects, suggesting the presence of two different binding sites for LA, one of which is intracellular (Sullivan et al., 1999). Downstream signaling induced by inositol trisphosphate or guanosine-5′-*O*-(3-thio)triphosphate (Sullivan et al., 1999) was not affected by LA (Nietgen et al., 1997), suggesting that the action of LA is on the receptor or coupled G protein. However, nonspecific membrane actions of LA are possible, and we did not directly confirm a protein site of action in our previous studies. Comparison of the effects of intracellular QX314 on m1 muscarinic and LPA receptors showed not only very similar calculated IC_{50} values, but also similar maximal degree of inhibition and slope of the inhibition curve (Hollmann et al., 1999), suggesting a common site of action. No similarity exists between the receptors, but because they couple to similar G proteins, we hypothesized that LA may inhibit G protein function.

In the present study we tested this hypothesis. Specifically, we 1) determined stereoselectivity of LA effect to investigate whether LA act on a protein site; 2) determined the G protein subtypes coupling to LPA, m1 muscarinic, trypsin, and angiotensin$_{1A}$ receptors; and 3) determined whether specific G protein α-subunits are involved in the LA effect.

Experimental Procedures

The studies were performed in *Xenopus laevis* oocytes. These cells express endogenous LPA and trypsin receptors; other G protein-

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ABBREVIATIONS: LA, local anesthetics; LPA, lysophosphatidic acid; MCh, acetyl-β-methylcholine bromide; AT, angiotensin.

coupled receptors can be expressed conveniently. Intracellular Ca^{2+} release as a response to receptor stimulation is easily assessed as Ca^{2+}-activated Cl^- currents. The size of the cells makes intracellular injection straightforward. In addition, using oocytes allowed comparison with our previous results obtained in this model. The study protocol was approved by the Animal Research Committee at the University of Virginia.

Materials. Molecular biology reagents were obtained from Promega (Madison, WI), and other chemicals were obtained from Sigma (St. Louis, MO). QX314 and the stereoisomers of ropivacaine were a gift from Astra Pharmaceuticals, L.P. (Westborough, MA).

Oocyte harvesting, receptor expression, electrophysiologic recording, and intracellular injections were performed as described previously (Durieux et al., 1993, 1994; Chan and Durieux, 1997; Nietgen et al., 1997; Sullivan et al., 1999).

Oligonucleotide Injection. Phosphorothioate oligonucleotides were synthesized by the University of Virginia Research Facility. The antisense sequences, shown in Fig. 2A, are complementary to specific 20-base segments with less than 50% homology with other types of *X. laevis* G_α proteins (from Shapira et al., 1999). Sense oligonucleotides were used as control. Uninjected oocytes (for experiments on the LPA or endogenous protease receptor) or those injected 24 h prior with cRNA encoding the m1 or AT_{1A} receptor were injected with 50 nl of sterile water containing 50 ng/cell antisense or sense oligonucleotides. Control cells were injected with the same amount of sterile water. Twenty-four and 48 h after oligonucleotide injection, the cells were tested as described below.

Analysis. Results are reported as mean ± S.E.M. At least 12 oocytes were used to determine each data point unless noted otherwise. As variability between batches of oocytes is common, responses were at times normalized to control response. Statistical tests used are indicated under *Results*. $p < 0.05$ was considered significant. Concentration-response curves were fit to the following logistic function, derived from the Hill equation: $y = y_{min} + (y_{max} - y_{min})\{1 - X^n/(X_{50^n} + x^n)\}$ where y_{max} and y_{min} are the maximum and minimum response obtained, n is the Hill coefficient, and X_{50} is the half-maximal effect concentration (EC_{50}) for agonist or the half-maximal inhibitory effect concentration (IC_{50}) for antagonist.

Results

LPA Responses in *X. laevis* Oocytes. To provide baseline measurements and to assure that our model behaved similar to our previous studies, we determined the concentration-response relationship for LPA. LPA induced inward currents as described previously by us (Durieux et al., 1992; Durieux and Lynch, 1993; Chan and Durieux, 1997) and others (Fernhout et al., 1992; Guo et al., 1996; Liliom et al., 1996; Kakizawa et al., 1998; Noh et al., 1998) (Fig. 1A). As shown in Fig. 1B, the response to LPA was concentration-dependent. Half-maximal effect concentration (EC_{50}) was $6.0 \pm 3.3 \times 10^{-7}$ M. Maximal responses of 4.3 ± 0.5 μC were obtained at a LPA concentration of 10 μM. Calculated E_{max} was 5.1 ± 0.5 μC and Hill coefficient was 0.57 ± 0.08. These findings compare closely with data reported in our previous studies (Durieux, 1995; Durieux and Nietgen, 1997; Nietgen et al., 1997, 1998).

Ropivacaine Stereoselectively Inhibits LPA Receptor Function. To determine whether LA inhibition of LPA signaling takes place at a protein site, we studied stereoselectivity of LA action. We selected *S*(−) and *R*(+)-ropivacaine for these experiments. Both stereoisomers concentration dependently inhibited functioning of LPA receptors (e.g., Figure 1C) activated by LPA at EC_{50} (6.0×10^{-7} M; Fig. 1D). Calculated IC_{50} for the clinically relevant *S*(−)-enantiomer of ropivacaine was $23.8 \pm 3.3 \times 10^{-3}$ M. *R*(+)-Ropivacaine showed an approximately 5-fold greater inhibitory potency than did *S*(−)-ropivacaine (IC_{50}, $4.8 \pm 0.2 \times 10^{-3}$ M); the difference in IC_{50} between *S*(−) and *R*(+) was significant ($p = 0.001$, *t* test), but we observed no statistically significant difference in Hill coefficients of both curves [1.2 ± 0.3 for *R*(+) versus 2.1 ± 0.4 for *S*(−), $p = 0.102$, *t* test]. These results demonstrate that LPA signaling is inhibited by ropivacaine enantiomers in a stereoselective manner. Although an action on an organized (and hence stereoselective) lipid membrane can not be ruled out by these experiments, the data are compatible with a protein interaction for ropivacaine, as suggested for other LA in our previous studies (Nietgen et al., 1997).

Our data show that *S*(−)-ropivacaine, the clinically relevant enantiomer, is approximately 6-fold less potent than bupivacaine and 4-fold less potent than lidocaine in blocking LPA signaling (Nietgen et al., 1997). Like bupivacaine, *S*(−)-ropivacaine inhibited in a noncompetitive manner (Fig. 1E). EC_{50} for LPA in the presence of *S*(−)-ropivacaine at IC_{50} (23.8×10^{-3} M) was $1.7 \pm 0.9 \times 10^{-7}$ M; this was not significantly different from the EC_{50} of $1.6 \pm 0.2 \times 10^{-7}$ M obtained under control conditions ($p = 0.744$, *t* test). In contrast, E_{max} was reduced by the presence of *S*(−)-ropivacaine from 1.1 ± 0.1 to 0.6 ± 0.03 μA ($p < 0.001$, *t* test).

Functional Degradation of G Protein α-Subunits by Injection of Antisense Oligonucleotides. Stereoselectivity of ropivacaine block is compatible with our hypothesis that G proteins are a target site in the inhibitory effect of LA on LPA signaling. We therefore determined whether receptors inhibited by LA share a common coupled Gα-subunit, by selectively depleting Gα proteins using antisense oligonucleotides directed against the G protein subunits. To verify that this system functioned appropriately in our hands, we determined the G protein α-subunits coupling to 1) endogenous protease receptors and 2) recombinantly expressed m1 muscarinic receptors in *X. laevis* oocytes. We studied these receptor systems previously (Hollmann et al., 1999), and the G protein α-subunits coupling to these receptors have been determined previously by others allowing verification of the technique (Shapira et al., 1999). Since the receptors of interest induce intracellular Ca^{2+} release, we used antisense oligonucleotides directed against $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_o$. Oocytes injected with vehicle served as control.

First, we determined the G protein α-subunits coupling to the endogenous protease receptor. We reported previously that the protease trypsin induces Ca^{2+}-activated Cl^- current in oocytes (Durieux et al., 1994). The site of action of trypsin was extracellular, the response desensitized completely, and the suggested mechanism was the presence of a protease receptor on the oocyte. As in our previous study, trypsin (1 μg/ml) induced responses in oocytes similar to those induced by LPA (data not shown). As shown in Fig. 2B, responses elicited by trypsin (1 μg/ml) 24 h after injection of antisense oligonucleotides were not affected by anti-$G\alpha_o$ (90 ± 19% of control response, $p > 0.05$; unless otherwise stated, all determinations of G protein α-subunits were compared by one-way analysis of variance with Dunnett correction) or anti-$G\alpha_{11}$ (81.3 ± 16.6% of control response, $p > 0.05$). In contrast, oocytes injected 24 h before measurement with anti-$G\alpha_q$ (32.4 ± 6.2% of control response, $p < 0.05$) or anti-$G\alpha_{14}$ (45.2 ± 8.6% of control response, $p < 0.05$) showed a signif-

icantly inhibited response to trypsin. Nearly identical results were obtained when experiments were performed 48 h after antisense injection (data not shown). These results indicate that the response evoked by trypsin is mediated by $G\alpha_q$ and $G\alpha_{14}$, suggesting strongly that the trypsin response is mediated by a G protein-coupled receptor system.

Next we investigated the G protein α-subunit coupling of m1 muscarinic responses elicited by stimulation with MCh at EC_{50} [5.7×10^{-7} M, calculated from our previous investigations (Hollmann et al., 1999)] in oocytes injected previously with m1 receptor cRNA. Experiments were performed 48 h after antisense injection. Injection of anti-$G\alpha_q$ (44.9 ± 8.5% of control response) or anti-$G\alpha_{11}$ (47.2 ± 8.3% of control response) affected MCh-induced responses significantly ($p < 0.05$), whereas anti-$G\alpha_o$-injected (92.2 ± 12.7% of control response) or anti-$G\alpha_{14}$-injected (98.9 ± 5.5% of control response) oocytes showed responses not significantly different from those observed in control cells ($p > 0.05$) (Fig. 2C). Our results show that muscarinic m1 signaling is mainly mediated by $G\alpha_q$ and $G\alpha_{11}$.

The studies using trypsin and muscarinic receptors demonstrate that Gα protein depletion by antisense oligonucleotides functions appropriately in our hands, as results are similar to those obtained by Shapira et al. (1999). These investigators demonstrated additionally that the reduced functional responses are associated with decreased mRNA and protein levels for the respective G proteins. After these confirmatory experiments, we proceeded to determine the Gα proteins mediating the LPA response.

$G\alpha_q$ and $G\alpha_o$ Mediate the Response to LPA in *X. laevis* Oocytes. To exclude the possibility that injection of DNA oligonucleotides per se affects responses to LPA (at EC_{50}, 6.0×10^{-7} M), we first studied the effects of sense oligonucleotide injection. Neither after 24 (data not shown) nor after 48 h (Fig. 2D) did sense-injected oocytes show responses different from those obtained in control oocytes

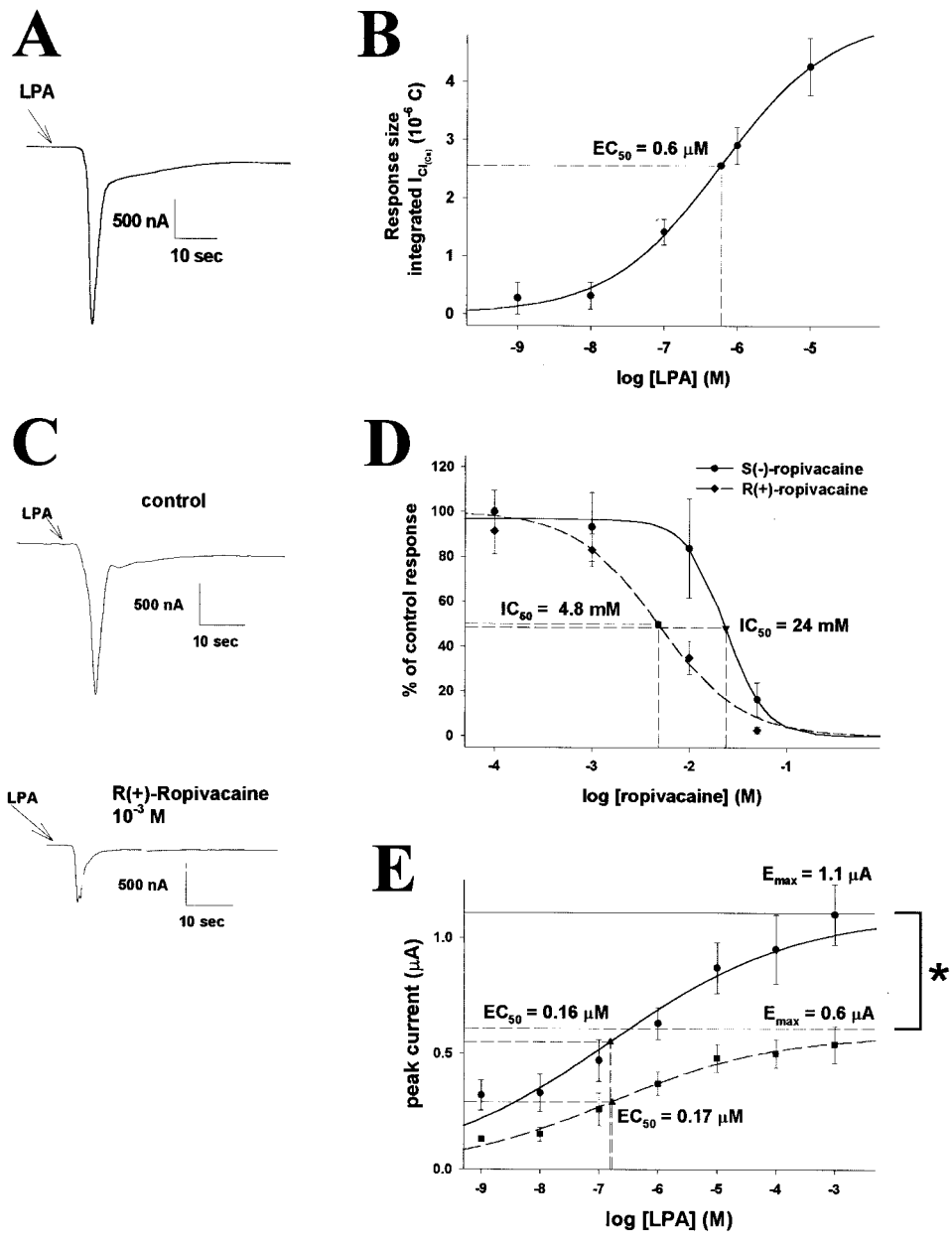

Fig. 1. Stereoselective inhibition of LPA signaling by ropivacaine. A, example of inward chloride current [$I_{Cl(Ca)}$] induced by LPA (0.6 μM) in oocytes expressing endogenous LPA receptors. B, LPA evokes $I_{Cl(Ca)}$ in a concentration-dependent manner. Curve fitting using the Hill equation revealed an EC_{50} of 6.0 ± 3.3 × 10^{-7} M. Maximal responses of 4.3 ± 0.5 μC were obtained with 10 μM LPA. Calculated E_{max} was 5.1 ± 0.5 μC. C, example trace of LPA-induced (0.6 μM) $I_{Cl(Ca)}$, under control conditions (top) and after a 10-min incubation in $R(+)$-ropivacaine (10^{-3} M) (bottom). D, both ropivacaine stereoisomers concentration dependently inhibit functioning of LPA receptors, activated by LPA at EC_{50} (0.6 μM), but with different half-maximal inhibition concentrations (IC_{50}). Calculated IC_{50} was 23.8 ± 3.3 × 10^{-3} M for $S(-)$-ropivacaine and 4.8 ± 0.2 × 10^{-3} M for $R(+)$-ropivacaine. Mean ± S.E.M. of control responses were 1.1 ± 0.3 μA for $S(-)$-ropivacaine and 2.3 ± 0.4 μA for $R(+)$-ropivacaine. E, $S(--)$-ropivacaine does not shift the LPA concentration-response curve to the right (−●−, control, EC_{50} 1.7 ± 0.9 × 10^{-7} M; ---, in the presence of ropivacaine 24 mM, EC_{50} 1.6 ± 0.2 × 10^{-7} M). Maximal effect concentration (E_{max}) was significantly reduced after $S(-)$-ropivacaine administration (1.1 ± 0.1 to 0.6 ± 0.03 μA), making a noncompetitive antagonism most likely.

injected with sterile water ($p > 0.05$). Thus, sense oligonucleotides have no effect.

Antisense injection 48 h (Fig. 2E) before oocytes were tested caused a significant ($p < 0.05$) inhibition of peak current when anti-Gα_o (38.1 ± 3.9% of control response) or anti-Gα_q (41.7 ± 4.9% of control response) were used. In contrast, injection of anti-Gα_{11} (102 ± 17% of control response) or anti-Gα_{14} (109 ± 21% of control response) was without significant ($p > 0.05$) effect compared with control oocytes (100 ± 19.3%). These findings indicate that LPA signaling is mediated primarily by Gα_q and Gα_o.

Since m1 muscarinic and LPA signaling are similarly inhibited by intracellular QX314, our findings suggest that the Gα_q protein, common to both signaling pathways, might be the target of the LA. This hypothesis was tested in the next series of experiments.

QX314 Inhibition of LPA Signaling Requires Gα_q. We investigated whether LA inhibit LPA signaling by an action on G protein α-subunits. To exclude an extracellular LA effect, we chose the permanently charged and therefore membrane-impermeant lidocaine analog QX314 for our experiments and applied it intracellularly. First, we determined the inhibition curve for intracellularly injected QX314 on LPA responses induced by stimulation with LPA (10^{-7} M). We chose an LPA concentration somewhat less than EC_{50} because injection of 150 nM KCl (50 nl) used in control cells and in treatment cells as buffer for QX314 caused substantially increased response sizes as a result of the increased intracellular Cl^- load. Figure 3A presents the results. Fitting to the Hill equation revealed a calculated IC_{50} of 424 ± 70 × 10^{-6} M, which is close to the value reported by Sullivan et al. (1999). Mean size of the control response was 3.6 ± 0.3 μA. To assure that QX314 effect is not dependent on response size, we also studied its action on responses elicited by a low concentration of LPA (1 nM). In control cells mean peak current was 1.1 ± 0.2 μA (Fig. 3B). Injection of QX314 at IC_{50} (424 × 10^{-6} M) caused an inhibition to approximately 38% (0.4 ± 0.1 μA), showing that the effect of the compound is independent of LPA response size.

We then tested the inhibitory effect of intracellularly injected QX314 at IC_{50} (424 × 10^{-6} M) on oocytes injected 48 h prior with anti-Gα_q or anti-Gα_o, which, as shown above, mediate the LPA response. Oocytes were stimulated with 0.1 μM LPA. As shown in Fig. 3C, significant inhibition of LPA responses by intracellularly injected QX314 was obtained only in anti-Gα_o-injected cells (0.7 ± 0.1 versus 2.0 ± 0.1 μA,

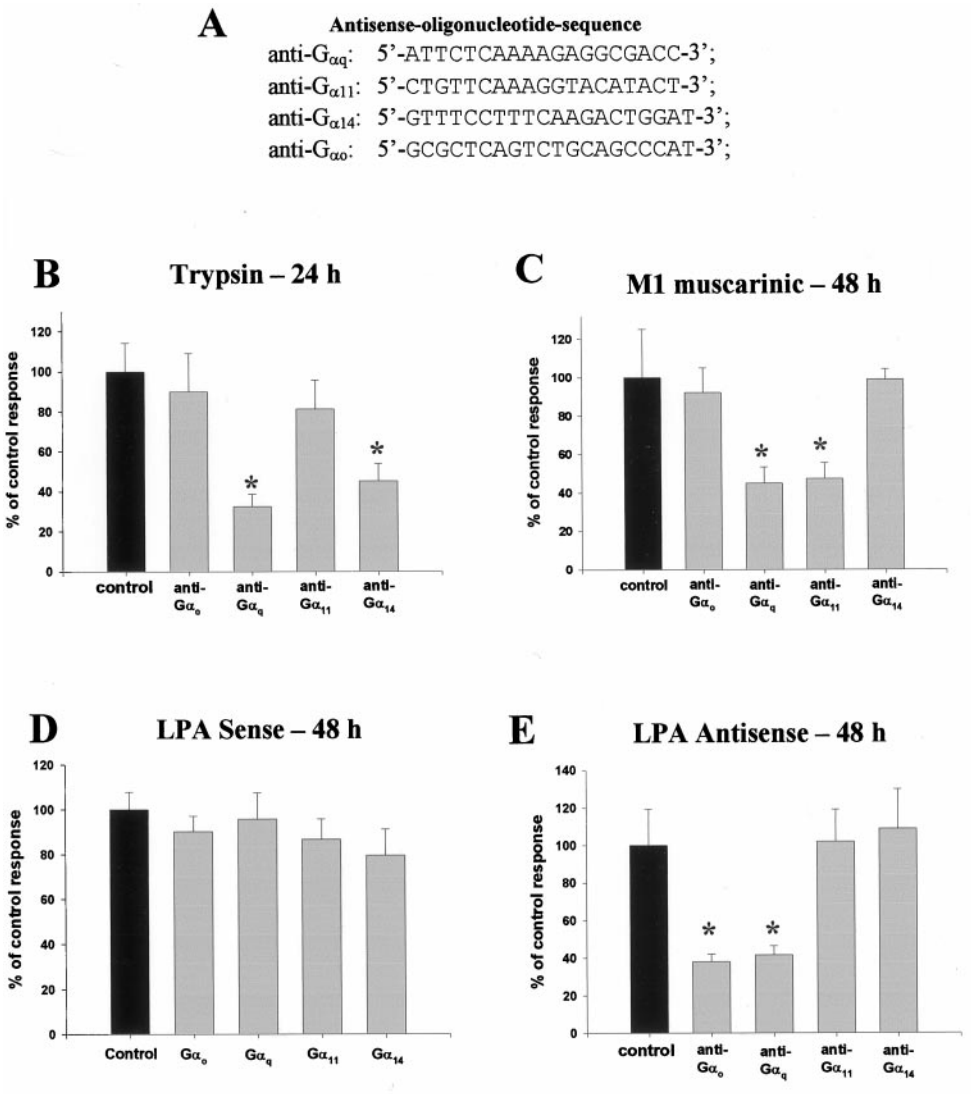

Fig. 2. Selective Gα-subunit depletion using antisense constructs. A, antisense oligonucleotide sequence used (Shapira et al., 1999). B, mean ± S.E.M. of peak currents of trypsin-induced (1 μg/ml) inward chloride current [$I_{Cl(Ca)}$] in control oocytes (filled column) and cells injected with DNA antisense oligonucleotides (50 ng/oocyte) targeted against specific Gα-subunits, 24 h before experiments. Mean ± S.E.M. of control currents was 1.4 ± 0.3 μA. Each data point includes at least 24 oocytes. C, MCh at EC_{50}-induced (5.7 ± 5.2 × 10^{-7} M) $I_{Cl(Ca)}$ in control oocytes (filled column, 0.4 ± 0.1 μA) and cells tested 48 h after injection of antisense constructs. D, effects of sense oligonucleotide (see Fig. 2A for sequences) injection 24 h prior on LPA signaling, stimulated by LPA at EC_{50} (6.0 ± 3.3 × 10^{-7} M). At neither time point did sense oligonucleotide-injected oocytes show a significant difference in response compared with sterile water-injected control oocytes [1.9 ± 0.1 μA (24 h) and 1.5 ± 0.2 μA (48 h)]. E, Mean ± S.E.M. of peak currents of LPA responses induced by LPA at EC_{50} (6.0 ± 3.3 × 10^{-7} M) in control oocytes (filled column, 1.8 ± 0.3 μA) and cells injected with the corresponding DNA antisense oligonucleotides (50 ng/oocyte) 48 h prior. Anti-Gα_o inhibited responses to 38.1 ± 3.9%, anti-Gα_q to 41.7 ± 4.9% as compared with control. In contrast, injection of anti-Gα_{11} (102 ± 17% of control response) or anti-Gα_{14} (109 ± 21% of control response) was without significant effect compared with control oocytes.

$p < 0.001$, t test). In contrast, in anti-Gα_q-injected cells QX314 had no significant ($p = 0.574$, t test) inhibitory effect (1.6 ± 0.2 versus 1.7 ± 0.2 μA). These findings indicate that QX314 inhibits only when functional Gα_q is present, suggesting that it mediates its inhibitory effect by acting on this G protein subunit.

In contrast to our findings, Noh et al. (1998) reported involvement of Gα_{11} in mediation of LPA responses in *X. laevis* oocytes. If so, LA inhibition of LPA signaling might also take place at this G protein. We therefore investigated the effect of intracellularly injected QX314 on either water- (control) or anti-Gα_{11}-injected oocytes (Fig. 3D). Oocytes, injected 48 h prior with water (control), showed an average peak current of 1.95 ± 0.15 μA after stimulation with LPA (10^{-7} M). Injection of QX314 at approximately IC_{50} (424×10^{-6} M) inhibited peak current of control oocytes to 63.1 ± 6.2% of control response. Anti-Gα_{11} injection changed neither the response to LPA (10^{-7} M) alone (96.4 ± 7.2% of control response) nor the effect of QX314 (66.4 ± 6.4% of control response in anti-Gα_{11}-injected oocytes) compared with control cells. Thus, in our hands at least, Gα_{11} seems not to be involved in mediation of the LPA response, and knockdown of Gα_{11} did not affect inhibition of the LPA response by QX314.

These results indicate that intracellularly injected QX314 acts by interference with G protein functioning and that its main target is the Gα_q-subunit, rather than Gα_o or Gα_{11}.

QX314 Inhibition of m1 Muscarinic Signaling also Requires Gα_q. If intracellular QX314 acts selectively on Gα_q, the m1 muscarinic receptor, which couples to this G protein, should be inhibited also by this LA. Since we determined that Gα_q and Gα_{11} are the primary G protein subunits coupling to the m1 muscarinic receptor, we studied the effect of intracellularly injected QX314 in oocytes expressing the m1 muscarinic receptor, 48 h after injection of antisense oligonucleotides directed against Gα_q or Gα_{11}. As shown above, injection of anti-Gα_q or anti-Gα_{11} alone reduced the control response (3.25 ± 0.19 μA) (Fig. 4A), elicited by stimulation of the m1 receptor with MCh (10^{-7} M), to 44 (1.44 ± 0.16 μA) and 48% (1.56 ± 0.24 μA), respectively. If QX314 acts on Gα_q, its half-maximal inhibition concentration should be independent of the receptor studied. We therefore used QX314 at IC_{50} as determined for LPA signaling. Intracellularly injected QX314 (424×10^{-6} M) had no significant ($p = 0.719$, t test, $n = 20$) effect in Gα_q-degraded oocytes (1.35 ± 0.19 μA, 94%), whereas 48 h after anti-Gα_{11} injection it inhibited responses to MCh (0.87 ± 0.15 μA) by the appro-

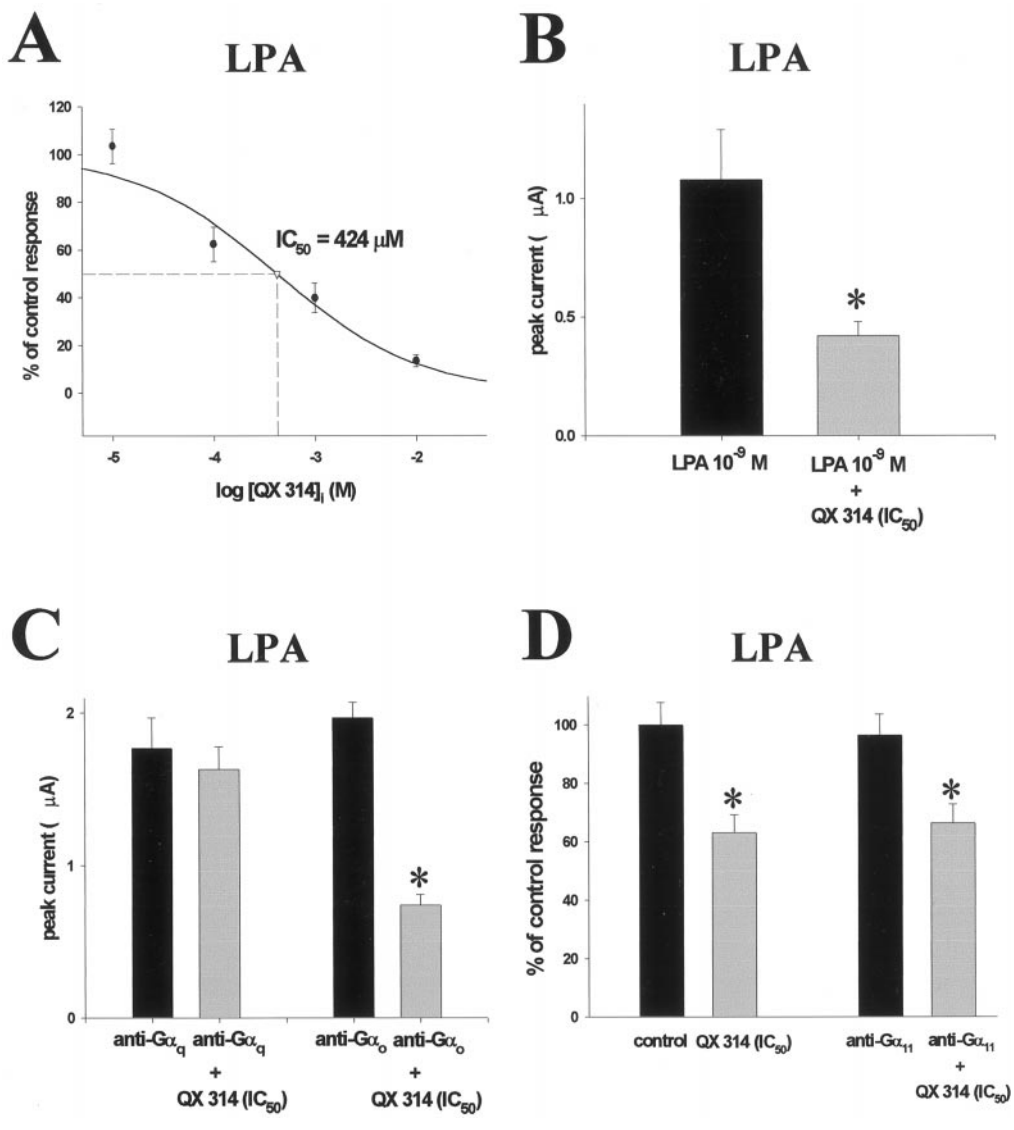

Fig. 3. Inhibition of LPA signaling by intracellular QX314 depends on the Gα_q-subunit. A, concentration-inhibition relationship for inhibition of LPA (10^{-7} M) inward chloride current [$I_{Cl(Ca)}$] by intracellularly injected QX314. Curve fitting using the Hill equation revealed an IC_{50} of 424 ± 70 × 10^{-6} M. Mean control response was 3.6 ± 0.3 μA. B, mean ± S.E.M. of peak $I_{Cl(Ca)}$ induced by stimulation with LPA (10^{-9} M) in either KCl-injected (50 nl of 150 mM KCl) control oocytes (filled column, 1.1 ± 0.2 μA) or cells injected with QX314 (at IC_{50}, 424 ± 70 × 10^{-6} M) (gray column, 0.4 ± 0.1 μA). C, mean ± S.E.M. of peak $I_{Cl(Ca)}$ for LPA responses, elicited by LPA (10^{-7} M) in oocytes injected with either anti-Gα_q or anti-Gα_o. Filled column, mean peak currents of control oocytes injected with 50 nl of 150 mM KCl; gray columns, mean peak currents of QX314-injected (at IC_{50}, 424 ± 70 × 10^{-6} M) cells. D, mean ± S.E.M. of peak $I_{Cl(Ca)}$, elicited by LPA (10^{-7} M) in oocytes injected with either sterile water or anti-Gα_{11} (filled columns, 1.95 ± 0.15 and 1.88 ± 0.14 μA, respectively). Gray columns, mean peak $I_{Cl(Ca)}$ of sterile water or anti-Gα_{11}-injected cells treated with QX314 (at IC_{50}, 424 ± 70 × 10^{-6} M) intracellularly.

priate percentage (55%, $p = 0.02$, t test, $n = 20$). Thus, QX314 inhibition is dependent on the presence of $G\alpha_q$, and the compound differentiates between two very similar G protein α-subunits: $G\alpha_q$ and $G\alpha_{11}$.

Trypsin Signaling is Inhibited by Intracellularly Injected QX314 and Lidocaine. To confirm that the $G\alpha_q$-subunit is an intracellular target site for LA, we studied the effect of intracellularly injected QX314 on responses induced by trypsin. Again, we used the IC_{50} for QX314 as determined for LPA signaling. Responses of the endogenous protease receptor, elicited by extracellular application of trypsin (1 μg/ml) to oocytes revealed a mean response size of 4.34 $\pm$ 0.46 μA (Fig. 4B). Ten minutes after injection of QX314 (424×10^{-6} M), mean response size was significantly ($p = 0.004$, t test, $n = 20$) reduced by 39% to 2.66 $\pm$ 0.3 μA. This finding indicates that another receptor coupled to $G\alpha_q$ is inhibited by intracellularly injected QX314, with similar potency as that observed at the LPA receptor. This supports our hypothesis that the $G\alpha_q$-subunit is likely to be an intracellular target site for LA.

To confirm that our findings determined for the quaternary lidocaine analog QX314 also hold for tertiary amide LA such as lidocaine, we next determined whether intracellularly injected lidocaine is able to inhibit trypsin-induced responses in the absence of $G\alpha_q$ or $G\alpha_{14}$, both of which were previously determined to be required for this signaling pathway. To prevent possible extracellular effects by lidocaine leaking to the outside, oocytes were superfused with Tyrode's solution at high flow rates (10 ml/min). As shown in Fig. 4C, 48 h after injection of anti-$G\alpha_q$ or anti-$G\alpha_{14}$ mean control response (3.49 $\pm$ 0.42 μA), elicited by extracellular application of trypsin (1 μg/ml) to oocytes expressing the endogenous protease receptor, was reduced to 33 (1.16 $\pm$ 0.27 μA) and 42% (1.46 $\pm$ 0.27 μA), respectively. Intracellularly injected lidocaine (445×10^{-6} M, approximate IC_{50} as determined from pilot studies) had no significant ($p = 0.294$, t test, $n = 24$) effect on $G\alpha_q$-degraded oocytes (1.58 $\pm$ 0.29 μA, 136%), whereas 48 h after anti-$G\alpha_{14}$ injection it inhibited responses to trypsin (0.42 $\pm$ 0.09 μA) by 71%, $p = 0.001$, t test, $n = 22$). Thus, inhibition by intracellular lidocaine is also dependent on the presence of $G\alpha_q$.

AT_{1A} Signaling is Primarily Mediated by $G\alpha_o$ and $G\alpha_{14}$. We showed previously (Nietgen et al., 1997) that AT_{1A} signaling is not inhibited by LA. If intracellular inhibition of G protein-coupled receptors by LA were due to action on the $G\alpha_q$-subunit, we would predict that AT_{1A} signaling is not primarily mediated by $G\alpha_q$ in our model. To test our hypothesis, we determined the G protein α-subunits coupling to the AT_{1A} receptor (Fig. 4C). In oocytes recombinantly expressing the AT_{1A} receptor, angiotensin II (10^{-6} M) induced an inward chloride current [$I_{Cl(Ca)}$] with an average peak current of 2.72 $\pm$ 0.29 μA, comparable with our previous data (Nietgen et al., 1997). Fourty-eight hours after antisense injection, oocytes injected with anti-$G\alpha_o$ (59.5 $\pm$ 7.4% of control re-

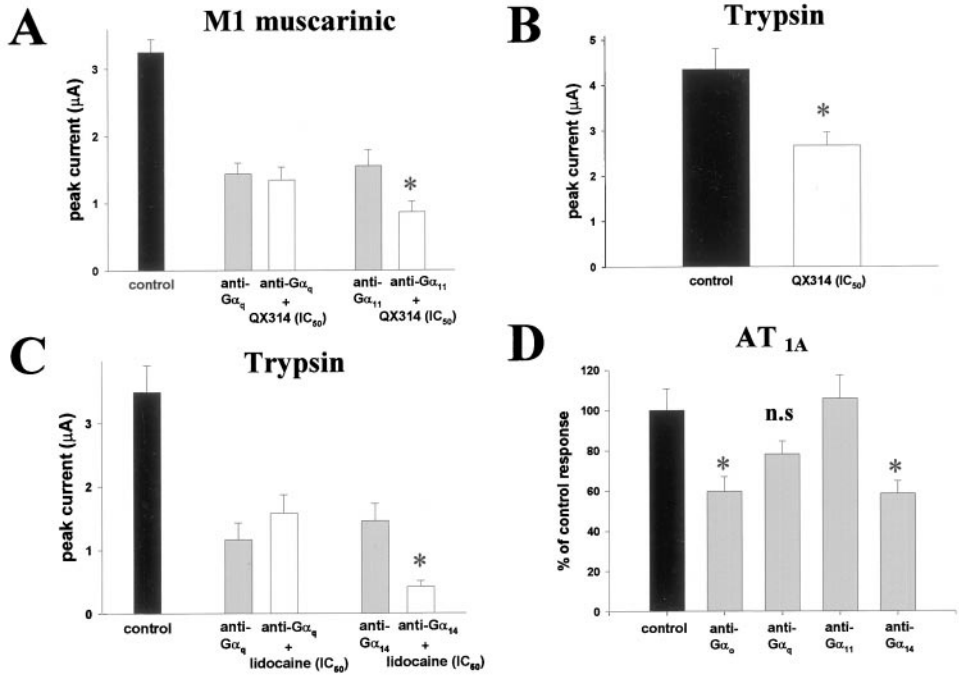

Fig. 4. Effects of local anesthetics and G protein antisense constructs on m1 muscarinic, protease, and AT_{1A} angiotensin receptors. A, mean $\pm$ S.E.M. of inward chloride current [$I_{Cl(Ca)}$], elicited by MCh (10^{-7} M) in oocytes, expressing m1 muscarinic receptors, injected with 50 nl of 150 mM KCl alone (control, filled column, 3.25 $\pm$ 0.19 μA), 50 nl of 150 mM KCl 48 h after injection of either anti-$G\alpha_q$ (gray column, 1.44 $\pm$ 0.16 μA) or anti-$G\alpha_{11}$ (gray column, 1.56 $\pm$ 0.24 μA), or QX314 (at IC_{50}, 424×10^{-6} M) 48 h after injection of either anti-$G\alpha_q$ (1.35 $\pm$ 0.19 μA) or anti-$G\alpha_{11}$ (0.87 $\pm$ 0.15 μA). B, mean $\pm$ S.E.M. of peak $I_{Cl(Ca)}$ induced by stimulation with trypsin (1 μg/ml) for either KCl-injected (50 nl of 150 mM KCl) control oocytes (filled column, 4.34 $\pm$ 0.46 μA) or cells injected with QX314 (at IC_{50}, 424×10^{-6} M) (open column, 2.66 $\pm$ 0.3 μA). C, mean $\pm$ S.E.M. of $I_{Cl(Ca)}$ elicited by trypsin (1 μg/ml) in oocytes, injected with 50 nl of 150 mM KCl (control, filled column, 3.49 $\pm$ 0.42 μA), 50 nl of 150 mM KCl 48 h after injection of either anti-$G\alpha_q$ (gray column, 1.16 $\pm$ 0.27 μA) or anti-$G\alpha_{14}$ (gray column, 1.46 $\pm$ 0.27 μA), or lidocaine (at IC_{50}, 445×10^{-6} M) 48 h after injection of either anti-$G\alpha_q$ (1.58 $\pm$ 0.29 μA) or anti-$G\alpha_{14}$ (0.42 $\pm$ 0.09 μA). D, mean $\pm$ S.E.M. of peak $I_{Cl(Ca)}$ induced by angiotensin II (10^{-6} M) in oocytes recombinantly expressing the AT_{1A} receptor. Average response in control oocytes (filled column) was 2.72 $\pm$ 0.29 μA. Cells injected with the corresponding DNA antisense oligonucleotides (50 ng/oocyte) 48 h prior revealed inhibition of peak current to 59.5 $\pm$ 7.4 of control response by anti-$G\alpha_o$ and 58.5 $\pm$ 6.3% of control response by anti-$G\alpha_{14}$, whereas injection of anti-$G\alpha_q$ (78.2 $\pm$ 6.4% of control response) or anti-$G\alpha_{11}$ (105.9 $\pm$ 11.4% of control response) was without significant effect compared with control oocytes.

sponse) or anti-Gα_{14} (58.5 ± 6.3% of control response) showed a significant ($p < 0.05$) reduction in peak current. In contrast, injection of anti-Gα_q (78.2 ± 6.4% of control response, $p > 0.05$) or anti-Gα_{11} (105.9 ± 11.4% of control response, $p > .05$) did not significantly affect responses elicited by stimulation with angiotensin II (10^{-6} M).

Thus, although a slight contribution of Gα_q cannot be ruled out, AT_{1A} receptor signaling is mediated primarily by Gα_o and Gα_{14}. Lack of LA effect on this receptor is therefore compatible with our hypothesis that intracellular inhibition of several G protein-coupled receptors by LA is due to interaction with the Gα_q-subunit.

Discussion

In the present study we have shown that LPA signaling is inhibited by ropivacaine stereoisomers in a concentration-dependent and stereoselective manner, strongly suggesting a protein site of action for ropivacaine. This inhibition is primarily due to a noncompetitive antagonism. We also found that LPA signaling is mediated primarily by Gα_q and Gα_o. Gα_q couples to LPA, muscarinic m1, and trypsin receptors and is a main target for intracellular LA inhibition of G protein-coupled receptors.

As in our previous studies, we used the *X. laevis* oocyte model. Several potential problems with the technique should be considered when interpreting the data. Using *X. laevis* oocytes requires performing experiments at room temperature, which raises the question whether the *X. laevis* LPA receptor and G proteins might behave differently from their mammalian orthologs. However, LPA-induced Ca^{2+} signaling in oocytes and in mammalian cells has been shown to be similar (Durieux et al., 1992; Durieux and Lynch, 1993; Moolenaar, 1995). We have only studied a single form of LPA signaling (Cl^- currents induced by intracellular Ca^{2+} release), whereas several intracellular signaling cascades are activated by LPA (e.g., decreases in cAMP, activation of Rho and ras). It is possible that these other actions might be affected differently by LA; indeed, this appears likely, as they involve different Gα-subunits in their signaling pathways (e.g., G_i mediates LPA-induced decreases in cAMP) (van Corven et al., 1989). Therefore, our data should not be extrapolated to LPA signaling in general. The m1 muscarinic and the AT_{1A} angiotensin receptor derive from rat and therefore were expressed at a lower temperature than they normally function in, but this has not been shown to affect their signaling properties appreciably. Although LPA and m1 muscarinic receptors have been shown to couple to G_q in mammalian cells, it is important to emphasize that the specificity of G protein coupling may depend on the cell type and species. However, 90% homology between mammalian and frog G proteins and lack of any evidence for differences in the physiological activity of species homologs of those G protein subunits make significant difference unlikely (Filtz et al., 1996). Despite these caveats, the oocyte model provides great advantages for studies of this kind. Particularly useful in the current context is the ability to study intracellular actions by microinjection of different compounds.

We supported our previous findings, which suggested that LA affect LPA signaling at either the G protein or the receptor itself (Nietgen et al., 1997; Sullivan et al., 1999) by showing stereoselectivity for ropivacaine inhibition of LPA signaling. This makes an interaction with a protein most likely. It should be realized, however, that phospholipids also contain a chiral carbon and that organized lipid membranes can show significant stereoselectivity (Dickinson et al., 1994). Interaction of LA with the compound LPA itself is unlikely because we demonstrated inhibition by intracellular QX314, whereas LPA acts extracellularly at its receptor (Sullivan et al., 1999). Our studies using ropivacaine revealed an additional finding with potential clinical relevance. We found (*S*-)-ropivacaine to be 6-fold less potent than bupivacaine and 4-fold less potent than lidocaine in inhibiting LPA signaling. Since LPA is likely to play a role in wound healing, LA, when injected around surgical wounds, may impair wound healing by inhibiting LPA signaling. If so, ropivacaine might have advantages over bupivacaine. Moreover, our findings with ropivacaine suggest that the LPA-inhibitory properties of racemic bupivacaine may largely reside in the clinically irrelevant stereoisomer dextrobupivacaine, suggesting that levobupivacaine would have fewer detrimental effects on wounds than the racemic preparation.

We found LPA signaling to be mediated mainly by Gα_o and Gα_q. In contrast, Noh et al. (1998) reported involvement of Gα_q and Gα_{11} in mediation of the LPA response in *X. laevis* oocytes. This inconsistency might be explained by the different antisense oligonucleotides used. Cross-degradation of other G protein α-subunits was not evaluated in Noh's study, whereas our experiments were performed with antisense sequences for which cross reactions with other G protein α-subunits have been determined on the mRNA and protein level (Shapira et al., 1999). In other words, whereas Noh et al. can not exclude that the reduction of LPA peak currents after injection of anti-Gα_{11} is in fact caused by degradation of [highly similar (Stehno-Bittel et al., 1995)] Gα_q, we can rule out that anti-Gα_{11} and anti-Gα_{14} had effects on the other G protein α-subunits. In addition, it is not surprising that they did not observe involvement of Gα_o in the LPA signaling pathway, because it was not, or only in small amounts, present in their oocytes.

Antisense results should not be overinterpreted in a quantitative manner. Specifically, although adding the percentage of inhibition obtained by anti-Gα_o and anti-Gα_q suggests complete inhibition of the LPA response when both G proteins are depleted, involvement of other G proteins can not be ruled out. For example, data from Shapira et al. (1999) would predict that combined Gα_q and Gα_{14} depletion would inhibit trypsin signaling by 140% (69% by anti-Gα_q and 68% by anti-Gα_{14}). In reality, when both antisense oligonucleotides were injected in combination, a 7% response to trypsin remained (Shapira et al., 1999). The underlying mechanisms may be several. Degradation of the G protein subunit may be incomplete, and the percentage of G protein α-subunit degradation may not necessarily correlate with the percentage of response inhibition. As stated by Shapira et al., it cannot be excluded that even residual amounts of any G protein can mediate a full response (Shapira et al., 1999). In addition, other G protein α-subunits, like Gα_{11} and/or Gα_{14}, which are usually not involved in the mediation of the LPA response when Gα_q and/or Gα_o are present, may be recruited when Gα_q and Gα_o are depleted. We attempted to determine the effect of combined injection of anti-Gα_q and anti-Gα_o, but most cells died and most surviving oocytes did not show a

stable holding potential of less than 0.5 μA. Shapira et al. (1999) reported similar difficulties.

Our previous studies (Hollmann et al., 1999; Sullivan et al., 1999) suggest that intracellular LA affect several G protein-coupled receptors with different structure (muscarinic m1 and LPA receptors) in a similar manner, making the G protein as a target most likely [because we have shown lack of interaction with the distal signaling pathway (Nietgen et al., 1997; Sullivan et al., 1999)]. Our results in the present study confirm this hypothesis. The significant inhibitory effect of intracellular QX314 on $G\alpha_o$-depleted (LPA signaling) and $G\alpha_{11}$-depleted (m1 muscarinic signaling) cells, and of intracellularly applied lidocaine in $G\alpha_{14}$-depleted (trypsin signaling) cells, contrasted with the lack of LA effect on $G\alpha_q$-degraded cells, suggests that LA might act intracellularly by inhibiting $G\alpha_q$ signaling. This is consistent with our findings that all three LA-sensitive receptors (muscarinic m1, LPA, and trypsin receptors) couple to $G\alpha_q$ and that those structurally completely different receptors are inhibited to a similar degree by intracellularly injected QX314. In contrast, the angiotensin 1_A receptor, which is not inhibited by LA, was found not to couple to $G\alpha_q$ to an appreciable degree.

Our results are consistent with findings by Xiong et al. (1999), who investigated LA inhibition of G protein-mediated modulation of potassium and calcium currents in anterior pituitary cells from rats. They demonstrated that lidocaine acts between agonist binding and G protein activation and concluded that such inhibition of G protein pathways might be an important component of the general action of LA (Xiong et al., 1999).

In conclusion, our study suggests that G protein-coupled receptors may be common targets for local anesthetics. The concentrations used in this study are routinely attained after local injection of these compounds. Inhibition of G protein-coupled receptors by LA results in part from an intracellular action, which can be largely explained by selective interference with $G\alpha_q$ function.

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