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Time-dependent Inhibition of G Protein–coupled Receptor Signaling by Local Anesthetics

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Background: **Several beneficial effects of local anesthetics (LAs) were shown to be due to inhibition of G protein–coupled receptor signaling. Differences in exposure time might explain discrepancies in concentrations of LAs required to achieve these protective effects *in vivo* and *in vitro* (approximately 100-fold higher). Using *Xenopus* oocytes and human neutrophils, the authors studied time-dependent effects of LAs on G protein–coupled receptor signaling and characterized possible mechanisms and sites of action.**

Methods: **Measurement of agonist-induced Ca^{2+}-activated Cl^- currents, using a two-electrode voltage clamp technique, and determination of superoxide anion production by cytochrome c assay were used to assess the effects of LAs on G protein–coupled receptor signaling in oocytes and primed and activated human neutrophils, respectively. Antisense knockdown of Gαq protein and inhibition of various proteins within the signaling pathway served for defining mechanisms and sites of action more specifically.**

Results: **LAs attenuated G protein–coupled receptor signaling in both models in a time-dependent and reversible manner (lidocaine reduced lysophosphatidic acid signaling to 19 ± 3% after 48 h and 25 ± 2% after 6 h of control response in oocytes and human neutrophils, respectively). Whereas no effect was observed after extracellularly applied or intracellularly injected QX314, a lidocaine analog, using Gαq-depleted oocytes, time-dependent inhibition also occurred after intracellular injection of QX314 into undepleted oocytes. Inhibition of phosphatases or protein kinases and agonist-independent G-protein stimulation, using guanosine 5'-O-3-thiotriphosphate or aluminum fluoride, did not affect time-dependent inhibition by LAs.**

Conclusion: **Inhibition of G protein–coupled receptor signaling by LAs was found to be time dependent and reversible. Critically requiring Gαq-protein function, this effect is located downstream of guanosine diphosphate–guanosine triphosphate exchange and is not dependent on increased guanosine triphosphatase activity, phosphatases, or protein kinases.**

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LOCAL anesthetics (LAs) have several beneficial effects[1–3] that cannot be explained by sodium channel blockade. Two examples are their antithrombotic[4] and antiinflammatory[5] actions, which are of potential importance for perioperative medicine. Interestingly, LAs exert these protective effects without compromising the physiologic function of these systems (*e.g.*, they do not induce bleeding[4] or impair the host defense[5]). Several lines of evidence suggest that interactions with G protein–coupled receptors may be of importance in these effects.

Studies performed to elucidate the cellular or molecular mechanisms of these actions have yielded contradictory results. Whereas LAs *in vivo* induced favorable effects at clinically relevant concentrations (1–10 μM),[1,5] *in vitro* concentrations approximately 100 times higher were required to achieve significant protective effects on platelet aggregation or inflammatory cells.[6] One obvious difference between *in vivo* and *in vitro* studies is the exposure time to the LAs: Exposure in most *in vitro* settings is 10–30 min, compared with hours or days *in vivo*. Therefore, time-dependent LA action might explain the discrepancy between *in vitro* and *in vivo* results. We therefore hypothesized that a prolonged exposure to LAs would increase their inhibitory potency, efficacy on G protein–coupled signaling pathways, or both. Clinically, LAs are frequently administered for long periods of time (days, in the case of epidural analgesia), and confirmation of this hypothesis might therefore have major implications for clinical use of these compounds. Initial support of our hypothesis came from Kohrs *et al.*,[7] who investigated the effects of LAs on coagulation *in vitro* and found that significant inhibition occurs only when blood is incubated with the LA for more than 60 min.

In addition to determining whether the effects of LAs are time dependent, we characterized possible sites and mechanisms of action. In a previous study, we demonstrated that inhibition of Gq-protein function is responsible for inhibition of G protein–coupled receptor signaling by LAs after brief exposure.[8] However, it cannot necessarily be assumed that time-dependent block takes place at the same site.

Therefore, in the current study, we determined the time-dependent effects of LAs on G protein–coupled receptor signaling, both in recombinant models and in human neutrophils. We found that signaling inhibition is profoundly time dependent and that an action on the Gq protein plays a major role.

Materials and Methods

After written informed consent to use human polymorphonuclear leukocytes (hPMNs) and approval of the study protocol by the Institutional Review Board and the Animal Care and Use Committee of the Universities of Virginia (Charlottesville, Virginia) and Maastricht (Maastricht, The Netherlands), the studies were performed in *Xenopus* oocytes and hPMNs. Oocytes express endogenous lysophosphatidic acid (LPA) receptors; other G protein–coupled receptors (*e.g.*, thromboxane A_2 [TXA_2] receptors) can be expressed conveniently. Intracellular Ca^{2+} release in response to receptor stimulation is easily assessed as Ca^{2+}-activated Cl^- currents. The size of the cells makes intracellular injection straightforward. hPMNs express endogenous LPA and platelet-activating factor (PAF) receptors. We used the cytochrome c–reduction assay to measure extracellular superoxide anion (O_2^-) production by activated hPMNs as described by our group previously.[9,10] O_2^- generation was measured spectrophotometrically, as the superoxide dismutase–inhibitable reduction of cytochrome c. Using oocytes and hPMNs allowed comparison with our previous results obtained in these models.

Oocyte Experiments

Oocyte Expression. Our methodology for oocyte harvesting and expression has been described previously.[11] Briefly, oocytes were obtained from *Xenopus laevis* frogs, defolliculated with collagenase, and injected with complementary RNA. Ca^{2+}-activated Cl^- currents, induced by inositol trisphosphate–mediated intracellular Ca^{2+} release, were measured using a two-electrode voltage clamp.

Drug Administration. Lysophosphatidic acid and U-46619, used as agonists for the LPA or TXA_2 receptor, were diluted in Tyrode solution to the required concentration and were superfused (3 ml/min) over the oocyte for 10 s. The oocyte was positioned close to the inflow tubing so that complete exposure to test solutions was obtained in 4.8 ± 0.4 s (n = 20). Responses were quantified by measuring peak current and are reported in microamperes.

For intracellular administration of QX314, a third micropipette was inserted into the voltage clamped oocyte. The micropipette was connected to an automated microinjector (Nanoject; Drummond Scientific, Broomall, PA). Under voltage clamp, 50 nl (approximately 10% of total oocyte volume) of a 150-mM potassium chloride solution was injected for determination of the control response; in the treatment group, we injected 50 nl potassium chloride solution containing QX314. Injection was followed by superfusion with Tyrode solution for 10 min, preventing an extracellular effect of any QX314 that leaked from the puncture site or through the membrane. The Ca^{2+}-activated Cl^- current was then induced by superfusion of LPA, as described previously.

Control, treatment, and at times recovery responses were obtained from different oocytes to prevent the effects of receptor desensitization from obscuring the results.

Oligonucleotide Injection. Phosphorothioate oligonucleotides were synthesized by the University of Virginia Research Facility (Charlottesville, Virginia). The antisense sequence is complementary to specific 20-base segments with less than 50% homology with other types of *X. laevis* Gα proteins.[12] Sense oligonucleotides were used as controls. Oocytes were injected with 50 nl sterile water containing 50 ng/cell antisense or sense oligonucleotides. Twenty-four hours after oligonucleotide injection, the cells were tested as described previously.

Experiments Using hPMNs

Preparation of hPMNs and measurements of O_2^- generation were undertaken according to the protocols previously published by our group.[9]

Materials

Molecular biology reagents were obtained from Promega (Madison, WI), and other chemicals were obtained from Sigma (St. Louis, MO). QX314 was a gift from Astra Pharmaceuticals, L.P. (Westborough, MA). Oocyte harvesting, receptor expression, and electrophysiologic recording were performed as described previously.[8,13,14]

Statistical Analysis

Results are reported as mean ± SD. For experiments in oocytes, at least 20 oocytes were used to determine each data point. Because variability between batches of oocytes is common, responses were normalized to control response. Statistical comparisons were made using an unpaired Student *t* test. For experiments with hPMNs, leukocyte metabolic activity is reported either as O_2^- production or as percentage change from control. Blood from at least 12 donors was used for each data point. Groups were compared using a paired Student *t* test. $P < 0.05$ was considered significant. SigmaStat 2.0 (Jandel Scientific Corporation, San Rafael, CA) was used for all statistical analyses. Time–inhibition curves were fit to a hyperbolic function: $y = y_{max} + (ab)/(b + x)$, where y_{max} is the maximal inhibition, a is $\max(y) - \min(y)$, b is the time to half maximal inhibition, and x is time.

Results

Before each experiment, concentration–response curves were determined to confirm half-maximal effect (EC_{50} for agonist) and inhibitory (IC_{50} for antagonist) concentrations published previously by our group (data not shown). We did not observe major changes in any

EC_{50} or IC_{50} as compared with our previous studies. EC_{50} and IC_{50} values used are referenced to the articles in which they were determined.

TXA_2 and LPA Signaling in Xenopus *Oocytes are Inhibited Time Dependently by LAs*

We studied TXA_2 signaling because of its involvement in platelet aggregation and LPA signaling because of its role in inflammatory processes.[9] Both have been shown to be inhibited by short-term exposure to LAs.[15–17] As LAs, we used bupivacaine (as the LA used most frequently for epidural anesthesia–analgesia) and lidocaine (as the LA used most commonly in the clinical and research settings). To determine time-dependent effects, we incubated oocytes in LA at one tenth of IC_{50} as determined for short-term exposure of each receptor signaling system and measured degree of inhibition at several time points up to 48 h. Cells incubated for the same periods in buffer served as controls.

Exposure to bupivacaine (1.2 μM)[15] for various durations induced a time-dependent inhibition (fig. 1A) of TXA_2 responses, evoked by stimulation of recombinantly expressed TXA_2 receptors with U-46619 at EC_{50} (0.32 μM).[15] Maximal inhibition was achieved after 48 h of incubation with bupivacaine, when thromboxane responses were inhibited to 25 ± 6% of control. Even more profound inhibition was obtained with bupivacaine (450 μM)[16] on LPA signaling (fig. 1B). LPA responses were elicited by stimulation of endogenous LPA receptors with LPA (at EC_{50}, 0.6 μM).[8] Complete inhibition of responses was already present after 8 h of incubation with bupivacaine. To exclude the possibility that time-dependent inhibition is restricted to bupivacaine, we next studied the effect of prolonged exposure to lidocaine (590 μM)[16] on LPA signaling. Responses evoked by stimulation with LPA (0.6 μM)[8] were inhibited after 48 h to 19 ± 3% of control responses (fig. 1C).

These results indicate that signaling of various G protein–coupled receptors is inhibited by LAs in a time-dependent manner and that, within 48 h after exposure, inhibitory potency is increased at least fourfold.

LPA- and PAF-induced Priming of hPMNs Is Inhibited by LAs in a Time-dependent Manner

To verify that the time-dependent effects observed are not specific to oocytes but also occur in human cells, we investigated the time-dependent inhibitory effects of LAs on the priming process of hPMNs. As agonists, we selected LPA and PAF, compounds that have been shown to induce priming and to be sensitive to LAs after brief exposure. *Priming* refers to a process where the response of hPMNs to a subsequent activating stimulus is potentiated. Release of oxygen metabolites is markedly enhanced when hPMNs have previously been primed.[18] The priming process has been shown to be a critical component of hPMN-mediated tissue injury both *in vitro* and *in vivo*.[18] As in our previous experiments, cells exposed to LAs were compared with control cells incubated for the same time period in buffer.

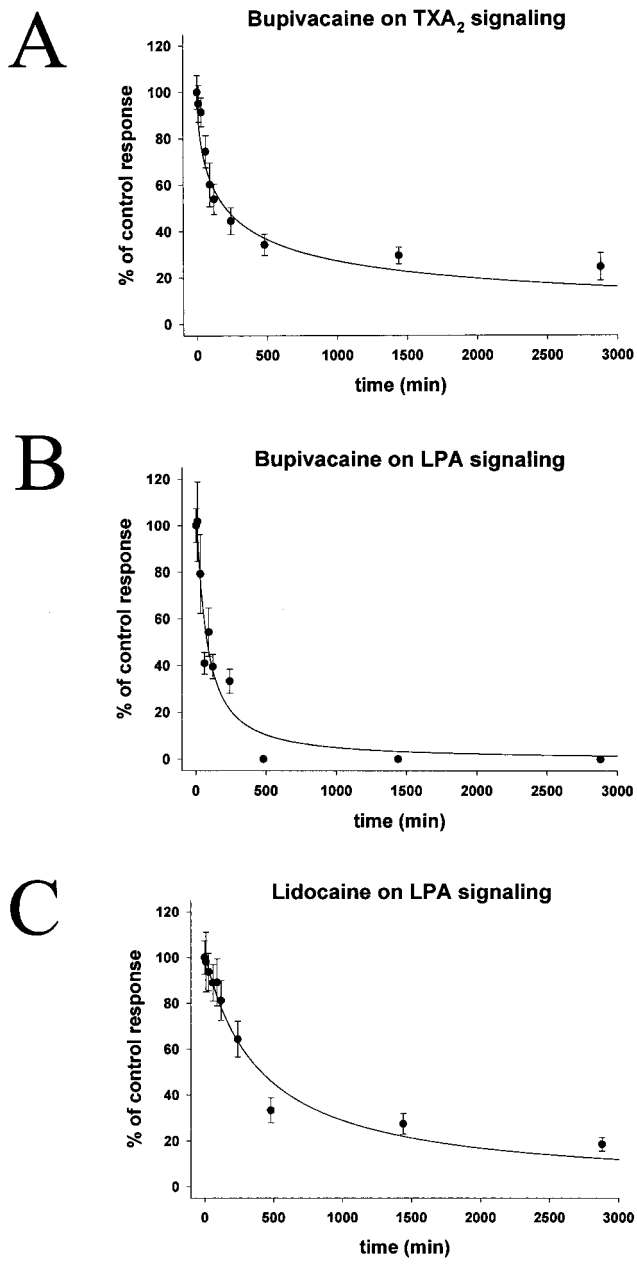

Fig. 1. Time-dependent inhibition of thromboxane A_2 (TXA_2) and lysophosphatidic acid (LPA) signaling by local anesthetics in *Xenopus* oocytes. (*A*) Time course for the effect of bupivacaine (1.2 μM) on TXA_2 signaling (n = 22). TXA_2 receptors were stimulated with U-46619 (specific TXA_2 receptor agonist, 0.32 μM). (*B*) Time course for the effect of bupivacaine (450 μM) on LPA signaling (n = 25). LPA responses were elicited by stimulation of endogenous LPA receptors with LPA (0.6 μM). (*C*) Time course for the effect of lidocaine (590 μM) on LPA (0.6 μM)–evoked responses (n = 25). Inhibition was determined at 15, 30, 60, 90, and 120 min and at 4, 8, 24, and 48 h and is shown as mean ± SD of responses (n ≥ 22 for each data point) normalized to control responses, incubated for the same duration in plain Barth solution.

As shown in figure 2A, 0.1 μM bupivacaine,[10] a con-

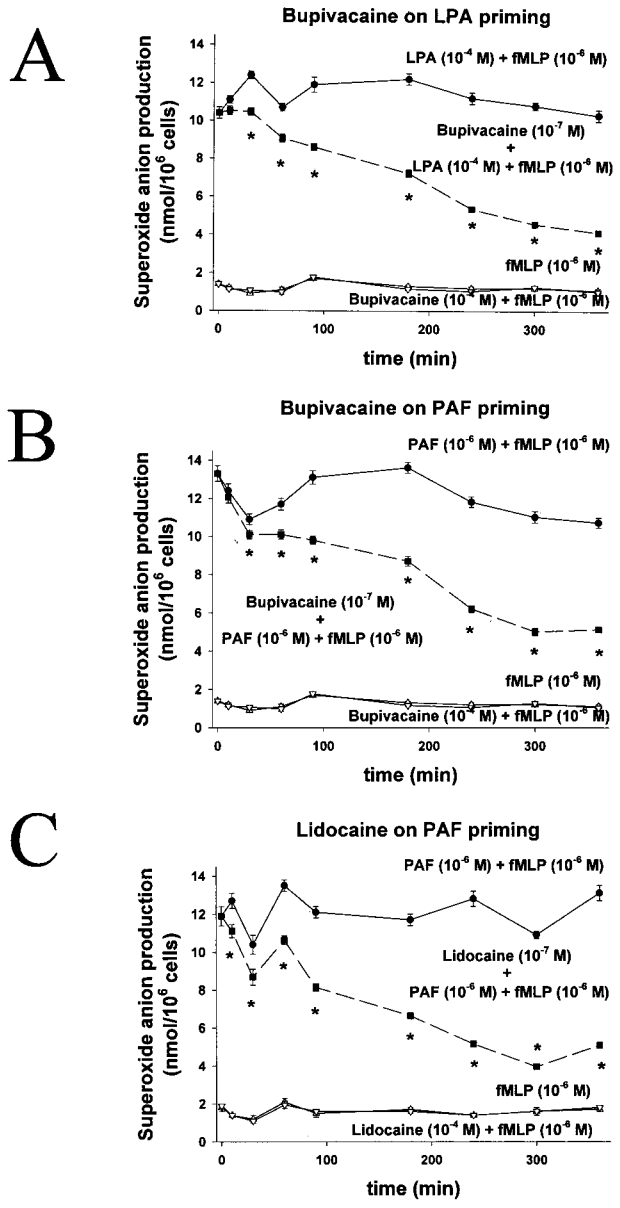

Fig. 2. Time-dependent inhibition of lysophosphatidic acid (LPA)– and platelet-activating factor (PAF)–induced priming of human neutrophils (hPMNs) by local anesthetics. (*A*) Time course for the inhibitory effect of bupivacaine (0.1 μM) on superoxide anion (O_2^-) production of hPMNs primed for 10 min with LPA (100 μM) and activated by f-methionine-leucine-phenylalanine (fMLP, 1 μM) (n = 14). (*B*) Time course for the inhibitory effect of bupivacaine (0.1 μM) on O_2^- production of hPMNs primed for 5 min with PAF (1 μM) and activated by fMLP (1 μM) (n = 12). (*C*) Time course for the inhibitory effect of lidocaine (0.1 μM) on O_2^- production of hPMNs primed for 5 min with PAF (1 μM) and activated by fMLP (1 μM) (n = 14). -●- = mean ± SD for O_2^- production of hPMNs primed with LPA (100 μM) or PAF (1 μM) and activated by fMLP (1 μM); -+- = mean ± SD for O_2^- production of hPMNs primed with LPA (100 μM) or PAF (1 μM) and activated by fMLP (1 μM) in the presence of the local anesthetic (bupivacaine or lidocaine, 0.1 μM); -△- = mean ± SD for O_2^- production of hPMNs solely activated by fMLP (1 μM); -▽- = mean ± SD for O_2^- production of hPMNs solely activated by fMLP (1 μM) in the presence of the local anesthetic (bupivacaine or lidocaine, 100 μM). O_2^- production was determined at 0, 10, 30, 60, 90, 180, 240, 300, and 360 min. * Significant ($P < 0.05$) difference compared with the control group (-●-).

centration 10–20 times less than that present in plasma after epidural administration,[19] significantly and time-dependently inhibited O_2^- production induced by priming with LPA (100 μM)[9] and activation with formyl-methionine-leucine-phenylalanine (fMLP, 1 μM).[9,10] After 6 h of exposure to LAs, responses were inhibited to 40 ± 1% of control. In contrast, fMLP (1 μM)[9,10]–induced activation of neutrophils in the absence of a priming agent was not affected by bupivacaine, even at a concentration of 100 μM, confirming that the action of the LA is on the priming and not on the activation process. To confirm that time-dependent inhibition of hPMN priming by LAs is independent of the priming agonist and LA chosen, we studied the effects of bupivacaine (fig. 2B) and lidocaine 0.1 μM (fig. 2C)[10] on priming induced by the inflammatory mediator PAF. PAF (1 μM)[10]–induced priming was also inhibited significantly and time dependently. After 6 h of incubation with bupivacaine (0.1 μM),[10] PAF-primed O_2^- production was reduced to 47 ± 1% of control. Lidocaine (0.1 μM)[10] inhibited PAF (1 μM)[10]–primed hPMN activation to 39 ± 3% of control after 5 h of exposure. Again, unprimed, fMLP (1 μM)[9,10]–induced superoxide release was at no time significantly inhibited, either by bupivacaine or by lidocaine (100 μM).

Taken together, these results show that time-dependent inhibition by LAs is not restricted to oocytes but also occurs in human cells.

Time-dependent Inhibition by LAs Is Reversible

To determine whether the time-dependent inhibition of hPMN priming is reversible, we exposed hPMNs to bupivacaine (1 μM; fig. 3A) or lidocaine (1 μM; data not shown) for 6 h. We chose a high LA concentration because we anticipated to have a greater likelihood of inducing irreversible effects. Subsequently, we washed the hPMNs for various durations in buffer, primed them with PAF (100 μM),[10] and activated them 5 min later with fMLP. O_2^- production after 6 h of incubation in bupivacaine (1 μM; fig. 3A) was inhibited to 29 ± 2% of control, which is comparable with the results reported above. After a 9-h washout, PAF-primed O_2^- production was still inhibited to 48 ± 2% of control. Similar results were obtained using lidocaine. The duration of the washout could not be extended beyond 9 h because of the limited survival of hPMNs under these experimental conditions. Therefore, we studied the reversibility of prolonged exposure to LAs in *Xenopus* oocytes. Oocytes were incubated for 48 h in 4.5 mM bupivacaine (fig. 3B) or 5.9 mM lidocaine (data not shown). Thereafter, the LA was washed out for various durations. Oocytes were then voltage clamped, and LPA (at EC_{50}, 0.6 μM)[8]–induced chloride currents were measured. Oocytes treated in the same manner but incubated in buffer only served as a control group. As shown in figure 3B, LPA responses were nearly abolished (3 ± 1% of control) after 48 h of

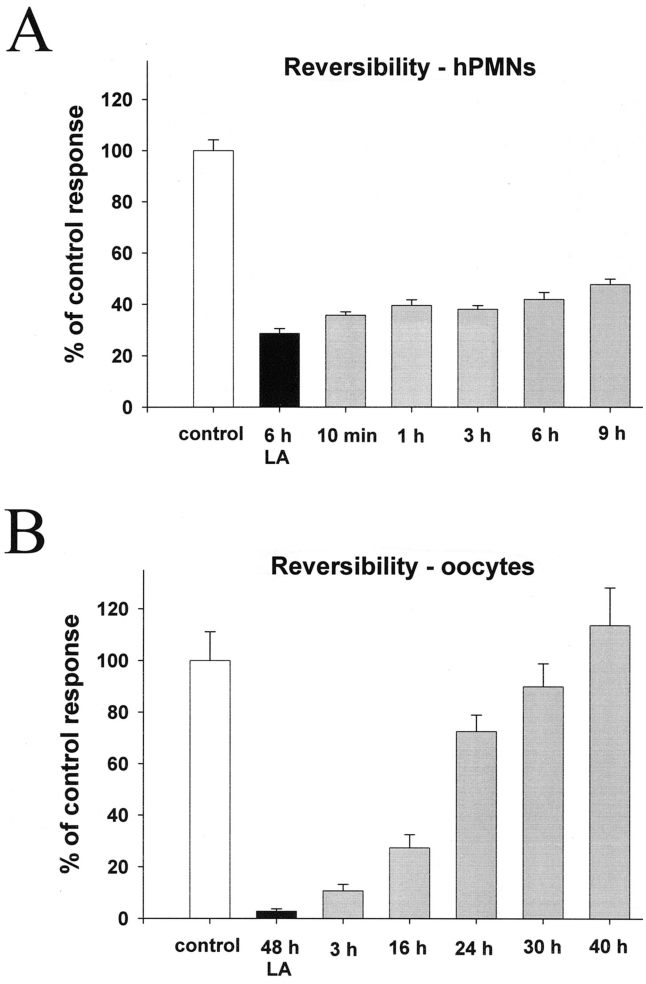

Fig. 3. Reversibility of time-dependent inhibition by local anesthetics (LAs). (*A*) Mean ± SD for superoxide anion production of human neutrophils (hPMNs) primed with platelet-activating factor (1 μM) and activated by f-methionine-leucine-phenylalanine (1 μM) after 6 h of incubation with bupivacaine (1 μM; *black bar*) and washout with plain buffer solution for various durations (*gray bars*) (n = 15). Data are normalized to corresponding control cells (*white bar*), which underwent exactly the same procedure solely in the absence of the LA. (*B*) Mean ± SD of lysophosphatidic acid responses in oocytes, elicited by stimulation with lysophosphatidic acid (0.6 μM) after 48 h of incubation with bupivacaine (4.5 mM; *black bar*) and washout with plain Barth solution for various durations (*gray bars*) (n = 22). Data are normalized to corresponding control responses (*white bar*), obtained from oocytes that underwent exactly the same procedure solely in the absence of the LA.

exposure to bupivacaine (4.5 mM). Complete reversibility of the effect of LA occurred only after a 40-h washout, indicating that time-dependent inhibition by LAs is reversible, but only after prolonged washout.

Time-dependent Inhibition by LAs in Xenopus *Oocytes Occurs Intracellularly and Is Gq Dependent*

We previously showed that the Gq protein is the site of action for short-term inhibition by LAs.[8] Therefore, we determined whether time-dependent inhibition of LPA signaling still occurs in the absence of Gq. We selectively depleted the Gq protein using antisense oligonucleotides directed against this G-protein subunit. Twenty-four hours after injection of the oligonucleotides, oocytes injected with anti-Gq showed a mean response size after stimulation with LPA (at EC_{50}, 0.6 μM)[8] of 0.83 ± 0.08 μA, as compared with 1.47 ± 0.08 μA in cells injected with the corresponding sense oligonucleotides. Seventy-two hours after anti-Gq injection, LPA responses of Gq-degraded oocytes were still reduced to 54 ± 8% of control (1.29 ± 0.11 *vs.* 0.69 ± 0.1 μA). This indicates that the antisense constructs appropriately reduced functional Gq-protein concentrations. As shown in figure 4A, depletion of Gq protein led to complete suppression of the effects of LA. LPA signaling was not significantly inhibited at any time point.

If the Gq protein is the major site of time-dependent inhibition, any long-term LA effects should occur intracellularly only. To assess whether the site of action for the observed time-dependent effects is located intracellularly, we injected the permanently charged and therefore membrane-impermeant lidocaine analog QX314 (at one tenth of IC_{50}, 42 μM)[8] into oocytes and determined the inhibitory effect over time. LPA responses, elicited by stimulation with LPA (at EC_{50}, 0.6 μM),[8] were inhibited in a time-dependent manner by QX314, with a maximal reduction in LPA signaling to 40 ± 7% of control after 24 h (fig. 4B). This suggests an intracellular site of action. To exclude the possibility of an additional extracellular site of action for QX314, we also studied the effect of extracellularly applied QX314 (5 mM) on Gq-depleted oocytes. Similar to our findings for short-term exposure to QX314,[17] LPA signaling was not inhibited by long-term application of extracellular QX314 (fig. 4C). An additional extracellular site of action, at least for charged LAs, can therefore be excluded. We next studied whether lidocaine (partially charged at pH 7.4) is able to activate an additional extracellular site of action for time-dependent inhibition. After confirming functional absence of the Gq protein, as described above, time-dependent inhibitory effects of extracellularly administered lidocaine (at one tenth of IC_{50}, 590 μM)[16] were determined. As shown in figure 4D, responses of Gq-depleted cells, elicited by stimulation with LPA (at EC_{50}, 0.6 μM),[8] were inhibited significantly to approximately 75% of control responses. However, this effect was not time dependent.

Together, these results indicate that the site of action for time-dependent inhibition by LAs is located intracellularly and that this effect is critically dependent on the Gq protein. The presence of either an extracellular or an intracellular site of action for uncharged LAs can be deduced from the findings using extracellular lidocaine. However, this site does not contribute to time-dependent effects.

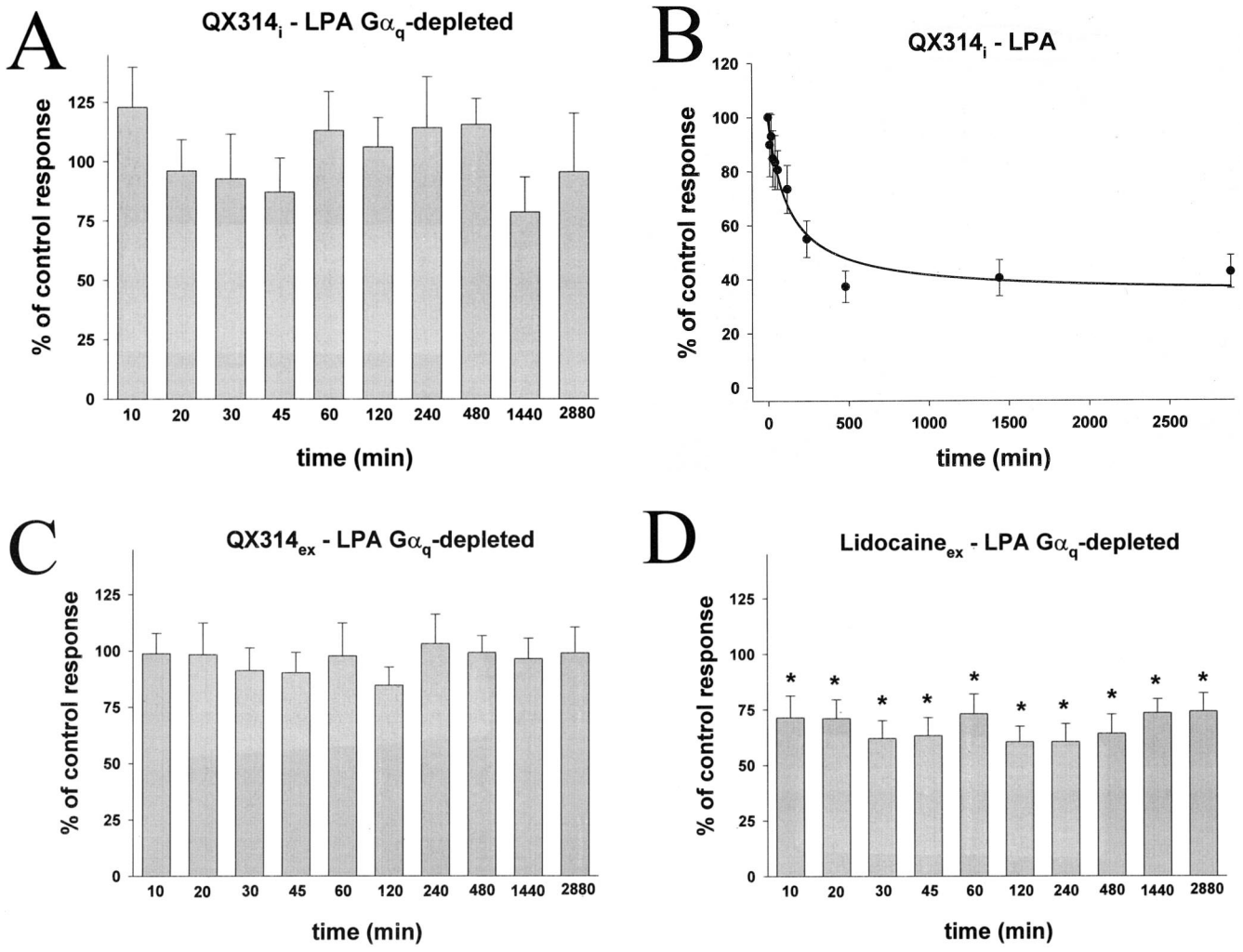

Fig. 4. Determination of the site of action for the time-dependent inhibition by local anesthetics in *Xenopus* oocytes. (*A*) Time course for the effect of intracellularly injected QX314 (42 μM) on lysophosphatidic acid (LPA) signaling after depletion of the Gαq protein using corresponding antisense oligonucleotides (n = 25). (*B*) Time course for the effect of intracellularly injected QX314 (42 μM) on LPA signaling in the presence of the Gαq protein (n = 25). (*C*) Time course for the effect of extracellular applied QX314 (42 μM) on LPA signaling in the absence of Gαq (n = 23). (*D*) Time course for the effect of extracellular applied lidocaine (590 μM) in Gαq-degraded oocytes (n = 24). LPA responses were elicited by stimulation of LPA receptors with LPA (0.6 μM). Inhibition was determined at time points indicated and is shown as mean ± SD of responses (n ≥ 22 for each data point) normalized to corresponding control responses, incubated for the same duration in plain Barth solution. * Significant ($P < 0.05$) difference compared with the control group.

PKC or Phosphatase Activity Is Not Required for Time-dependent Inhibition by LAs

To address the mechanisms underlying the time-dependent inhibition described above, we studied the role of protein kinase C (PKC) and phosphatases in time-dependent inhibition by LAs. We chose an 8-h period of LA incubation because maximal inhibition occurred in our previous experiment after this time period (*cf.* fig. 1B). PKC or phosphatase inhibitors were added to oocytes 1 h before incubation in LA. We investigated the effects of 10 μM chelerythrine (a PKC antagonist targeted to the substrate binding site of PKC) on LPA (at EC_{50}, 0.6 μM)[8]–evoked responses. As shown in figure 5A, inhibition of PKC increased LPA signaling significantly by approximately 40% of control response after 9 h (1-h pretreatment and 8-h study period) of incubation. The presumed mechanism is inhibition of receptor phosphorylation. Bupivacaine (at one tenth of IC_{50}, 450 μM)[16] inhibited LPA responses time dependently, with a maximal reduction to 13 ± 3% of control after 8 h. In the additional presence of chelerythrine (10 μM), the inhibition curve was shifted upward but otherwise similar to that obtained in the absence of the PKC antagonist. Maximal inhibition after 8 h was 20 ± 5% of control. Similar effects were observed using 10 μM bisindolylmaleimide (a selective competitive PKC antagonist for adenosine triphosphate binding to the catalytic domain). Bisindolylmaleimide significantly enhanced the LPA response to approximately 120% of control (fig. 5B). Bupivacaine (at one tenth of IC_{50}, 450 μM),[16] in either the absence or the presence of bisindolylmaleimide, inhibited LPA signaling in a time-dependent manner. The

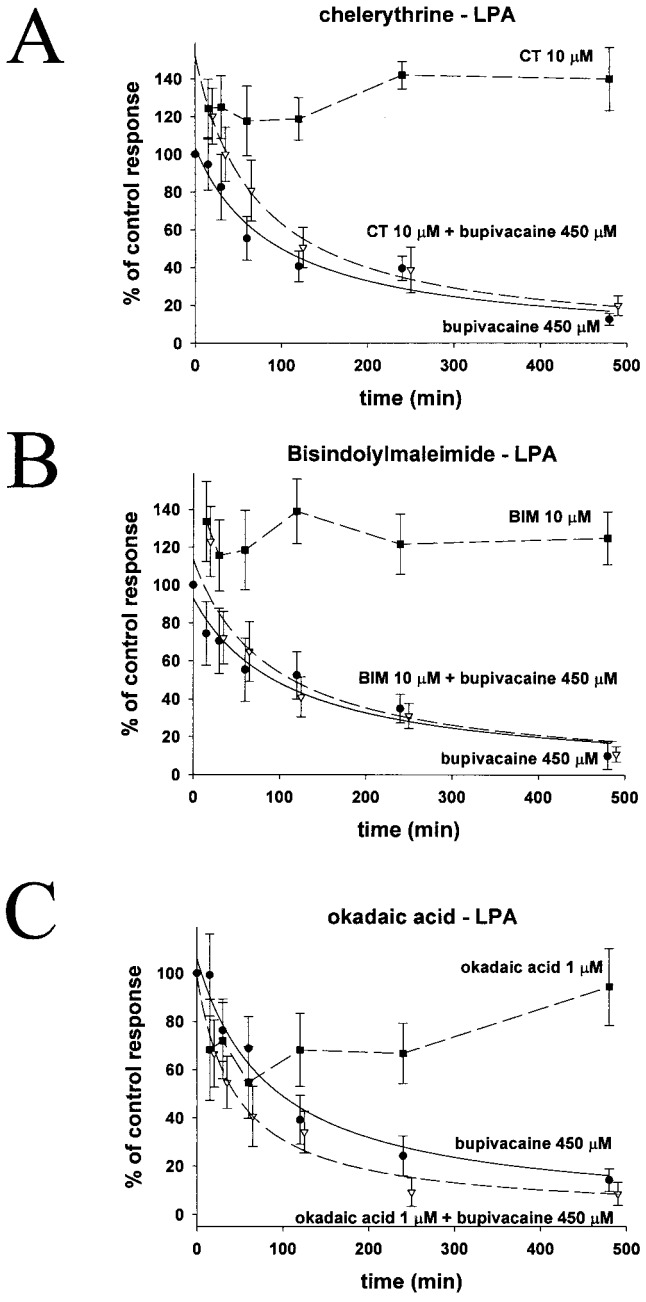

Fig. 5. Effects of protein kinase C and phosphatase inhibitors on time-dependent inhibition by local anesthetics in *Xenopus* oocytes. Time courses for the effects of chelerythrine (CT, 10 μM) (*A*; n = 22), bisindolylmaleimide (BIM, 10 μM) (*B*; n = 23), and okadaic acid (1 μM) (*C*; n = 22) on lysophosphatidic acid (LPA) signaling in the absence and presence of bupivacaine (450 μM). LPA responses were elicited by stimulation of LPA receptors with LPA (0.6 μM). Inhibition was determined at 15, 30, 60, and 120 min and at 4 and 8 h and is shown as mean ± SD of responses (n ≥ 22 for each data point) normalized to control responses, incubated for the same duration in plain Barth solution. -●- = time–inhibition curves for bupivacaine (450 μM) alone; -+- = inhibition by the protein kinase C (10 μM) or phosphatase (1 μM) antagonist alone; -▽- = inhibitory effect by the combination of bupivacaine (450 μM) and protein kinase C (10 μM) or phosphatase (1 μM) antagonist.

inhibition curves were virtually superimposable. Maximal inhibition after 8 h was 10 ± 7% of control response (bupivacaine alone) and 11 ± 4% of control response (bisindolylmaleimide plus bupivacaine). These findings suggest that time-dependent inhibition of receptor signaling by LA is not mediated by modulation of PKC signaling.

Comparable results were found for the phosphoserine–phosphothreonine phosphatase inhibitor okadaic acid. Okadaic acid, 1 μM, significantly decreased LPA signaling to approximately 70% of responses obtained for the control group (fig. 5C). This is most likely because of inhibition of receptor dephosphorylation. Bupivacaine (at one tenth of IC_{50}, 450 μM)[16] time-dependently inhibited LPA responses to 14 ± 5% of control and 9 ± 4% of control in the absence and presence, respectively, of okadaic acid. In addition, we studied a second selective protein phosphatase inhibitor, cantharidin (10 μM), and determined the inhibitory effect of bupivacaine (at one tenth of IC_{50}, 450 μM)[16] on LPA signaling in the presence of cantharidin after 8 h of incubation. LPA responses were inhibited to 5 ± 3% of control response. From these findings, we conclude that time-dependent inhibition by LAs is not dependent on either PKC or protein phosphatase activity.

GTPγS and Aluminum Fluoride–induced Chloride Currents in Xenopus *Oocytes Are Inhibited by LAs in a Time-dependent Manner*

Finally, to narrow down the site of action for LAs at the G protein, we investigated the time-dependent effects of bupivacaine (at one tenth of IC_{50}, 450 μM)[16] on guanosine 5'-O-3-thiotriphosphate (GTPγS) or aluminum fluoride–induced chloride currents in *Xenopus* oocytes. Both compounds induce irreversible G-protein activation because they cannot be hydrolyzed by guanosine triphosphatase activity. Whereas G-protein activation by GTPγS requires release of guanosine diphosphate (GDP) from the G protein, activation by aluminum fluoride does not. Therefore, the compounds can be used to determine whether an effect on G-protein function depends on guanosine diphosphate–guanosine triphosphate (GTP) exchange. As shown in figure 6A, chloride currents elicited by injection of GTPγS (1 mM) were inhibited in a time-dependent manner after incubation in bupivacaine (at one tenth of IC_{50}, 450 μM).[16] Maximum inhibition to 29 ± 10% of control response occurred after 4 h. Similar effects were obtained with aluminum fluoride (100 μM)–induced signaling (fig. 6B). Suppression to 17 ± 8% of control was observed after 4 h of treatment with bupivacaine (at one tenth of IC_{50}, 450 μM),[16] suggesting that time-dependent inhibition by LAs does not depend on interference with GDP–GTP exchange or increased guanosine triphosphatase activity.

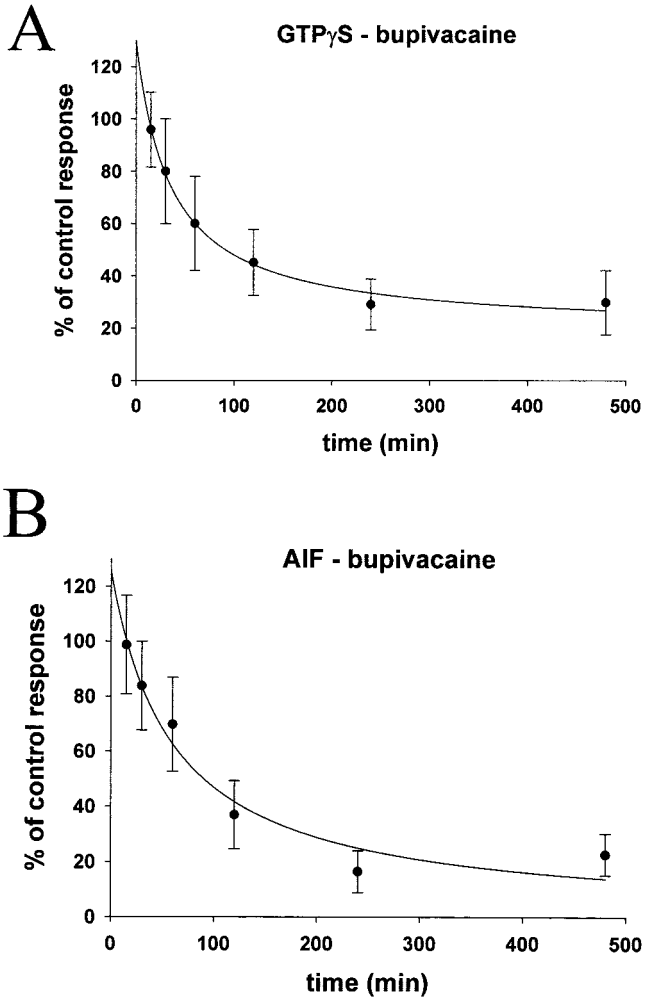

Fig. 6. Time-dependent inhibition of guanosine 5'-O-3-thiotriphosphate (GTPγS)– and aluminum fluoride (AlF)–induced chloride currents by local anesthetics in *Xenopus* oocytes. Time course for the effect of bupivacaine (450 μM) on GTPγS (1 mM; *A*)– and aluminum fluoride (100 μM; *B*)–induced chloride currents. Inhibition was determined at 15, 30, 60, and 120 min and at 4 and 8 h and is shown as mean ± SD of responses (n ≥ 25 for each data point) normalized to control responses, incubated for the same duration in plain Barth solution.

Discussion

The current study shows that G protein–coupled receptor signaling is inhibited by LAs in a time-dependent manner, both in *Xenopus* oocytes and in human neutrophils. This effect is reversible after prolonged (40-h) washout. In addition, we demonstrate that time-dependent inhibition by LAs requires functional Gq protein and does not result from interference with GDP–GTP exchange or increased guanosine triphosphatase activity. Furthermore, the effect is dependent neither on PKC nor on protein phosphatase activity.

As in our previous studies, we chose the *Xenopus* oocyte model for our mechanistic investigations. Several potential caveats of the technique have been discussed extensively in our previous articles and should be considered when interpreting the data.[8,11,13,14] However, 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 and the long survival time of these cells.

As G protein–coupled receptors, we used TXA_2, LPA, and PAF receptors. We chose them for several reasons. First, they have been shown to play major roles in coagulation (TXA_2[20–22]) and inflammation (LPA[9] and PAF[10,18]). These are the systems we are most interested in because of their great importance for perioperative medicine and because beneficial actions of LAs have been demonstrated in diseases mediated by these systems.[1,5] Second, we selected these receptors because we have shown previously that their signaling is inhibited by LAs after short-term exposure.[9,10,15–17]

To exclude the possibility that any effects observed are specific to oocytes, we supplemented our oocyte investigations with studies in human neutrophils. We observed time-dependent inhibition by LAs in both models. We believe that this effect may explain the discrepancy between the concentrations required to achieve various beneficial effects of LAs *in vitro* (short-term exposure to LAs) compared with *in vivo* settings (long-term exposure to LAs). However, several alternative or additional explanations for this discrepancy may exist and should be considered. LAs might interfere with coagulation or inflammatory cascades at several sites of action. If so, studies of single sites of action *in vitro* will never replicate the potency as determined *in vivo*. It is also conceivable that LAs might specifically prevent overstimulation of the coagulatory or inflammatory systems without impairing the physiologic function of these systems. Because most *in vitro* studies use blood from healthy volunteers or from animals under physiologic conditions, such effects might not be observed *in vitro*. This latter hypothesis is supported by the clinical observation that patients receiving LAs do not experience increased bleeding nor do they have increased susceptibility to infection, *i.e.*, despite the documented actions of these compounds on coagulation and inflammation, normal function of these systems is not affected. In addition, we have shown that epidural anesthesia with LAs selectively prevents surgery-induced hypercoagulation, without interfering with normal coagulation.[23] Also, in the current study, we observed that LAs had no effect on the activation process of hPMNs, even after prolonged LA incubation. Only priming, a process that has been shown to be a critical component of hPMN-mediated tissue injury both *in vitro* and *in vivo*[18] and that reflects excessive stimulation of the inflammatory cascade, was significantly and time-dependently inhibited by LAs.

We observed reversibility of time-dependent inhibition by LAs only after prolonged (40-h) washout, which is in contrast to our findings for short-term (10-min) exposure

to LAs where complete recovery was obtained after 5–10 min of washout.[14,11,16] This suggests different mechanisms of action. Specifically, both the long time course of the effect and the long washout required suggest that the time-dependent effect of LAs might depend on changes in protein expression. Our findings suggest that Gq might be the down-regulated target. However, solely complete loss of Gq-protein function would only explain an approximately 50% inhibition of signaling transduction. An additional effect on the common pathway further downstream of the Gq protein, for which the Gq protein is required (*e.g.*, changes in phospholipase C function) must be assumed.

To determine whether the Gq protein is required for time-dependent actions of LAs, we used the same antisense approach that we used previously, when we determined that the Gq protein is the site of action for short-term inhibition by LAs of G protein–coupled receptor signaling.[8] It is important to emphasize that both the current and the previous study were performed using frog Gq proteins and that species differences might exist. However, we have meanwhile shown that the inhibition of Gq-protein function by short-term exposure to LAs is not restricted to frog G protein but also holds true for its mammalian ortholog.[24]

The mechanism by which prolonged exposure to LAs interferes with Gq function is unclear. We found that the time-dependent inhibition of receptor signaling by LAs is independent from PKC and protein phosphatase activity and that the site of action must be downstream of GDP–GTP exchange because GTPγS- and aluminum fluoride–induced chloride currents were inhibited by LAs in a similar manner. These findings are compatible with down-regulation of the Gq protein by prolonged exposure to LAs.

In conclusion, our study suggests that G protein–coupled receptor signaling, which is involved in many processes relevant for perioperative medicine (*e.g.*, coagulation and inflammation), is inhibited by LAs in a time-dependent and reversible manner. This effect is not dependent on PKC or phosphatases but instead depends critically on Gq-protein function and is located downstream of GDP–GTP exchange.

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