Anesthesiology 2003; 99:1093–1101

Lidocaine Enhances $G\alpha_i$ Protein Function

Claudia Benkwitz, M.D., James C. Garrison, Ph.D.,† Joel Linden, Ph.D.,‡ Marcel E. Durieux, M.D., Ph.D.,§ Markus W. Hollmann, M.D., Ph.D.‖*

Background: **Local anesthetics inhibit several G protein–coupled receptors by interaction with the $G\alpha_q$ protein subunit. It is not known whether this effect on G protein function can be extrapolated to other classes of G proteins. The authors investigated interactions of lidocaine with the human adenosine 1 receptor (hA1R)–coupled signaling pathway. Activated A1Rs couple to adenylate cyclase *via* the pertussis toxin sensitive $G\alpha_i$ protein, thereby decreasing cyclic adenosine monophosphate formation. A1Rs are widely expressed and abundant in the spinal cord, brain, and heart. Interactions of LAs with the hA1R-coupled transduction cascade therefore might produce a broad range of clinically relevant effects.**

Methods: **The function of hA1Rs stably expressed in Chinese hamster ovary cells was determined with assays of cyclic adenosine monophosphate, receptor binding, and guanosine diphosphate/guanosine triphosphate $\gamma^{35}S$ exchange by using reconstituted defined G protein subunits. Involvement of phosphodiesterase and $G\alpha_i$ was characterized by using the phosphodiesterase inhibitor rolipram and pertussis toxin, respectively.**

Results: **Lidocaine (10^{-9}–10^{-1} M) had no significant effects on agonist or antagonist binding to the hA1R or on receptor–G protein interactions. However, cyclic adenosine monophosphate levels were reduced significantly to 50% by the LAs, even in the absence of an A1R agonist or presence of an A1R antagonist. This effect was unaffected by rolipram (10 μM), but abolished completely by pretreatment with pertussis toxin, which inactivates the $G\alpha_i$ protein. Therefore, the main target site for LAs in this pathway is located upstream from adenylate cyclase.**

Conclusions: **Lidocaine potentiates $G\alpha_i$-coupled A1R signaling by reducing cyclic adenosine monophosphate production. The study suggests an interaction site for LAs in a $G\alpha_i$-coupled signaling pathway, with the $G\alpha_i$ protein representing the prime candidate. Taken together with previous results showing inhibitory LA interactions on the $G\alpha_q$ protein subunit, the data in the current study support the hypothesis that specific G protein subunits represent alternative sites of LA action.**

* Resident and Research Fellow, Department of Anesthesiology, University of Virginia; Resident and Research Fellow, Department of Anesthesiology, University of Wuerzburg, Wuerzburg, Germany. † Professor and Chair, Department of Pharmacology, University of Virginia. ‡ Professor, Departments of Internal Medicine and Physiology, University of Virginia. § Professor, Department of Anesthesiology, University of Virginia; Professor, Department of Anesthesiology, University Hospital Maastricht, Maastricht, The Netherlands. ‖ Resident and Assistant Professor in Research, Department of Anesthesiology, University of Virginia; Resident and Assistant Professor in Research, Department of Anesthesiology, University Hospital Maastricht, Maastricht, The Netherlands; Resident and Assistant Professor in Research, Department of Anesthesiology, University of Heidelberg, Heidelberg, Germany.

Received from the Department of Anesthesiology, University of Virginia, Charlottesville, Virginia. Submitted for publication May 21, 2002. Accepted for publication June 16, 2003. This work was supported in part by departmental funding (Department of Anesthesiology, University of Wuerzburg, Wuerzburg, Germany) and by the 2000 Ben-Covino Research Award, sponsored by AstraZeneca Pain Control of Sweden. This work was awarded the second prize for the Resident's Research Essay Contest at the Annual Meeting of the American Society of Anesthesiologists in Orlando, Florida, October 12–16, 2002. Presented in part at the 75th IARS Clinical and Scientific Congress in Fort Lauderdale, Florida, March 17, 2001, and at the 2001 annual meeting of the American Society of Anesthesiologists in New Orleans, Louisiana, October 13, 2001.

Address reprint requests to Dr. Durieux: Department of Anesthesiology, University of Virginia, P. O. Box 800710, Charlottesville, Virginia, 22908-0710. Address electronic mail to: med2p@virginia.edu. Individual article reprints may be purchased through the Journal Web site, www.anestheiosology.org.

ALTHOUGH local anesthetics (LAs) are considered primarily Na^+-channel blockers and are traditionally used for local and regional anesthesia or antiarrhythmic treatment, they exert significant effects in other clinical settings. These alternative actions cannot be attributed primarily to Na^+-channel blockade, but result most likely because LAs interact with other cellular systems.[1–4] Such effects range from neuroprotection, reduction of hypnotic requirements, and inhibition of bronchial hyperresponsiveness, to the treatment of tinnitus, migraine, and pruritus. Of particular interest are reports indicating that LAs modulate the inflammatory response. *In vivo* they reduce reperfusion injury in the brain, lung, and heart. Systemically or topically applied LAs were shown to be effective in the treatment of inflammatory diseases of the gastrointestinal tract[5,6] and to shorten the duration of postoperative ileus in patients undergoing abdominal surgery.[7,8] Furthermore, LAs were reported to reduce microvascular permeability, a property that could account for the therapeutic effects of topically or systemically applied LAs in cases of peritonitis and severe burns. LAs were also attributed to antithrombotic effects, because epidural anesthesia is known to reduce postoperative thromboembolic complications.[9–11] Even effects that have been classically considered to result from Na^+-channel blockade (*e.g.*, antiarrhythmic, negative inotropic, and cardiotoxic LA effects) in part might result from interactions at other targets.[12,13]

Unfortunately, the molecular mechanisms behind these alternative and potentially beneficial LA effects are poorly understood. In addition to the well-characterized Na^+-channel block (EC_{50}, 50–100 μM), LAs show a variety of molecular effects at concentrations obtained routinely in clinical practice,[1] including inhibitory actions on ion channels[14–17] and on several Ca-signaling G protein–coupled receptors, such as lysophosphatidate,[18–20] thromboxane,[21,22] platelet-activating factor,[23] prostaglandin E_2,[24] and muscarinic m1 and m3 receptors.[25,26] Recent investigations from our laboratory have shown that the inhibitory actions of LAs on G protein–coupled signaling cascades take place at different locations: on the extracellular and intracellular domains of the receptor and intracellularly on the G protein itself. For some signaling pathways (*e.g.*, lysophosphatidate and muscarinic m1 and m3 signaling), extracellular and intracellular effects were superadditive. This resulted in half-maximal inhibitory concentrations for LAs in the nM range (*i.e.*, 1,000-fold less than those required for neuronal Na^+-channel blockade).[19,25–27] In contrast to the variable extracellular actions of LAs (most likely explained by the highly diverse structures of the different recep-

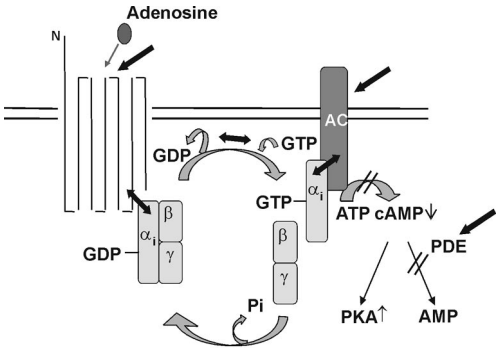

Fig. 1. A schematic diagram of the adenosine 1 receptor–coupled signaling pathway with receptor, G protein cycle, and effector. Binding of $G\alpha_i$ reduces adenylate cyclase (AC) activity resulting in decreased cyclic adenosine monophosphate (cAMP) synthesis (cAMP ↓). *Bold black arrows* indicate potential interaction sites of lidocaine with this pathway. AMP = adenosine monophosphate; GDP = guanosine diphosphate; GTP = guanosine triphosphate; PDE = phosphodiesterase; Pi = phosphate; PKA = cAMP-dependent protein kinase.

tors studied), intracellular inhibition of these signaling pathways by LAs is similar among these receptors and depends on the $G\alpha_q$ protein subunit.[27] For example, the angiotensin$_{1A}$ receptor, which couples to $G\alpha_o$ and $G\alpha_{14}$ (but not $G\alpha_q$) in the same system (*Xenopus* oocytes), was found not to be affected by LAs.[18] These findings suggest that direct interactions of LAs with specific G proteins might represent an important alternative LA action.

Based on amino acid homology, at least three other G protein subfamilies, in addition to the $G\alpha_q$ family, have been identified so far, coupling to different receptors and effector structures and exhibiting different activities. These additional subfamilies include G_{12}, the cholera toxin-sensitive $G\alpha_s$ family ($G\alpha_s$ and $G\alpha_{olf}$), and the pertussis toxin (PTX)–sensitive $G\alpha_i$ ($G\alpha_i$ and $G\alpha_o$).[28] So far no data exist on interactions of LA with other families of G proteins. We therefore investigated the effects of lidocaine on the human adenosine 1 receptor (hA1R), which couples to the $G\alpha_i$ protein. This pathway was chosen for several reasons. First, $G\alpha_i$ is structurally and functionally completely different from the $G\alpha_q$ family, making it an interesting target for comparison. Second, hA1Rs couple (*via* $G\alpha_i$) to adenylate cyclase, thereby reducing cytoplasmic cyclic adenosine monophosphate (cAMP) levels[29] (fig. 1), and LA effects on the complete hA1R signaling cascade can be studied conveniently by cAMP assays in A1R-transfected Chinese hamster ovary (CHO) cells. CHO cells represent one of the most commonly used and well-characterized cellular models. This cell line has been used extensively for the expression of numerous receptors, including the adenosine receptor by us and others.[29–37] Third, of the four G protein–coupled adenosine receptors cloned thus far, the A1R is best suited for studies of reconstitution with recombinant G protein subunits,[30] allowing for detailed investigation of LA effects on specific G protein functions. Fourth, A1R is the best characterized member of the purinergic receptor family. It is expressed widely, but predominantly in the brain, spinal cord, and heart.[38] Its activation has been implicated in a great variety of physiologic functions, including sedation, anticonvulsant activity, analgesia, and neuroprotection, and it is associated with negative chronotropic, dromotropic, and inotropic responses.[39] Possible interactions with local anesthetics therefore might be of clinical relevance.

We addressed the following questions: Does lidocaine modulate the hA1R-coupled signaling pathway? What are the LA interaction sites? Our findings indicate that lidocaine potentiates the hA1R signaling pathway by facilitating the ability of activated $G\alpha_i$ to inhibit adenylate cyclase. In view of the inhibitory actions of LAs on $G\alpha_q$, this demonstrates that LA modulation of G protein function shows subfamily dependence. This in part may provide the molecular basis for some of the alternative clinical effects of LA, which cannot be attributed primarily to Na^+-channel blockade.

Methods

cDNAs, Expression Vector, and Cell Model

Human A1R cDNA was subcloned into the pDoubleTrouble vector, and CHO-K1 cells were transfected as described previously, using the calcium phosphate precipitation method.[40] Forty-eight hours after the transfection, cells were reseeded in Dulbecco's Modified Eagle Medium with nutrient mixture F12 (DMEM/F12 1:1) supplemented with 10% (v/v) fetal calf serum and with 2 mg/ml G-418. Transfected clones were selected by virtue of their viability in the neomycin analogue G-418 and carried in 0.6 mg/ml G-418. Expression of the receptors was verified by ligand binding and adenylate cyclase assays (see Adenylate Cyclase Assays section).

Cell Culture

CHO-K1 cells stably transfected with the hA1R were routinely cultured in DMEM/F12, supplemented with 10% (v/v) fetal calf serum, penicillin (100 U), streptomycin (100 μg/ml), and 0.6 mg/ml G-418. Cells were grown at 37°C in a humidified 5% CO_2/95% air atmosphere and subcultured two or three times weekly using trypsin (0.05% w/v)/ethylenediaminetetraacetic acid (EDTA) (0.02% w/v).[31]

Adenylate Cyclase Assays

For the measurement of hA1R-mediated cAMP accumulation, cells were grown as a confluent monolayer in 200-ml flasks. The culture medium was discarded, and the cells were washed once with phosphate-buffered saline (PBS) and then incubated in 5 ml PBS with 5 mM EDTA for 5–10 min. Dissociated cells were transferred to 30 ml PBS and centrifuged for 3 min at 1,000 rpm. The cell pellet was resuspended at a density of 100,000 cells/200 μl in N-(2-hydroxyethyl)piperazine-N-'(2-eth-

anesulfonic acid) (HEPES) (20 mM, pH 7.2), buffered DMEM/F12 containing 1 U/ml adenosine deaminase (ADA). Cells were allowed to recover for 30 min at room temperature. Drugs were added in a volume of 0.05 ml medium containing 1 U/ml ADA, the adenylate cyclase activator forskolin (final concentration, 10 μM), and, in some experiments, the phosphodiesterase inhibitor rolipram (final concentration, 10 μM). Incubation continued for 10 min in a 37°C shaking water bath, and then reactions were stopped by adding 0.5 ml 0.15 N HCl. Tubes were centrifuged for 10 min at 5,000 rpm and 0.5 ml was removed for measurement of cAMP levels, by the University of Virginia Diabetes Core Lab, using a radioimmunoassay method as described by Harper and Brooker.[41] Cells used for cAMP determination had a viability of more than 95% as assessed by the exclusion of trypan blue.

Membrane Preparation for Binding Experiments and Guanosine Diphosphate/Guanosine Triphosphate $\gamma^{35}S$ Exchange Assays

Cells from at least 10 confluent dishes (20 cm in diameter) were washed twice with ice cold PBS, scraped into ice cold buffer A (10 mM HEPES, 10 mM EDTA, 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each of leupeptin, pepstatin A, and aprotinin, pH 7.4), and homogenized. The homogenate was centrifuged at 34,000*g* for 20 min at 4°C and then washed three times in ice cold buffer HE (10 mM HEPES, 1 mM EDTA, 0.02% [w/v] NaN_3, 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each of leupeptin, pepstatin A, and aprotinin, pH 7.4). The pellet was resuspended in HE buffer plus 10% (w/v) sucrose at a concentration of 5 mg/ml, dounce homogenized, and stored in aliquots at −80°C.[31] Protein concentration was determined by the Lowry method using bovine serum albumin as the standard and by linear regression.

Membrane preparations used for guanosine diphosphate (GDP)/guanosine triphosphate (GTP) $\gamma^{35}S$ exchange assays were in addition treated with urea (*i.e.*, urea stripped membranes) to remove all endogenous G proteins of the CHO cells that would have interfered with the reconstitution of A1R with recombinant G protein subunits. Cells were treated exactly as described in the previous section, except that after the first centrifugation step, cells were resuspended in HE buffer with 7 M urea and incubated 30 min on ice followed by centrifugation at 142,000*g* for 30 min at 4°C. The pellet was then washed three times and finally resuspended in HE buffer plus 10% (w/v) sucrose. Aliquots at a concentration of 5 mg/ml were stored at −80°C.

Binding Experiments

The levels of expression of A1Rs in stably transfected CHO cells were determined in saturation binding experiments using the specific A1R antagonist [^{3}H]-8-cyclopentylxanthine (CPX). Increasing concentrations of [^{3}H]-CPX (0.1–10 nM) were incubated with 20 μg of membrane protein in a total volume of 100 μl HE buffer (10 mM HEPES, 1 mM EDTA, 0.02% NaN_3, and 1 U/ml ADA, pH 7.4) for 90 min at room temperature. By using a Brandel cell harvester (Brandel, Gaithersburg, MD), membranes were collected onto Whatman GF/C glass fiber filters by washing them three times with ice cold binding buffer (10 mM Tris and 5 mM $MgCl_2$, pH 7.4). Radioactivity trapped on filters was measured by using a scintillation counter (Beckman LS6500; Beckman Coulter, Inc., Fullerton, CA). Nonspecific binding was measured in the presence of 100 μM 8-cyclopentyltheophylline (CPT). All reactions were performed in triplicate. Specific binding was fit to a single-site binding model using nonlinear curve fitting to calculate receptor density expressed as the maximum binding capacity (B_{max}) and the dissociation constant K_d (Prism 3.0; GraphPad Software, San Diego, CA).[31]

To investigate interactions of lidocaine with agonist or antagonist binding, competition binding experiments were performed. To determine interactions with specific binding of the antagonist [^{3}H]-CPX, membrane protein aliquots (20 μg) were incubated with various concentrations of lidocaine (10^{-9}–10^{-2} M) and with [^{3}H]-CPX (2 nM) in a total volume of 100 μl HE buffer (10 mM HEPES, 1 mM EDTA, 0.02% NaN_3, and 1 U/ml ADA, pH 7.4) for 90 min at room temperature. Radioligand binding was determined as described in the previous section, by using a cell harvester, glass fiber filters and by measuring radioactivity in a scintillation counter. To determine interactions with specific binding of the agonist [^{125}I]-N^6-aminobenzyladenosine ([^{125}I]-ABA), 20 μg of membrane protein was incubated with various concentrations of lidocaine (10^{-10}–10^{-2} M) and with [^{125}I]-ABA [1 nM] in HE buffer (10 mM HEPES, 1 mM EDTA, 10 mM $MgCl_2$, 0.02% NaN_3, and 1 U/ml ADA, pH 7.4) for 180 min at room temperature. Radioligand binding was again determined as described in the preceding section for [^{3}H]-CPX, except that [^{125}I]-ABA was counted dry in a gamma counter.

Preparation of Recombinant $G\alpha_{i1\beta1\gamma2}$

$G\alpha_i$ and $\beta\gamma$ subunits were expressed using the baculovirus/Sf9 insect cell system and purified to homogeneity as described previously.[42,43]

Reconstitution of hA1R with G Proteins and GDP/GTP $\gamma^{35}S$ Exchange Assays

Urea stripped membrane protein (approximately 200 fmol measured as [^{3}H]-CPX binding sites) was centrifuged for 10 min at 12,000 rpm in a microcentrifuge at 4°C. The pellet was resuspended in 450 μl reconstitution buffer (25 mM HEPES, 100 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 0.1% bovine serum albumin, 1 μM adeno-

sine-5'-[(β,γ)-imido]triphosphate, 1 mM dithiothreitol, and 1 U/ml ADA) and incubated 30 min on ice. After 30 min, $G\alpha_{i1}$ and $\beta_{1\gamma2}$ dimers and GDP were added, so that the final concentrations in a volume of 500 μl were as follows: 20 nM $G\alpha_{i1}$, 40 nM $\beta_{1\gamma2}$, 500 nM GDP. The receptor–G protein mixture was incubated another 30 min on ice, followed by a 10-min equilibration at 25°C. The assay was started by the addition of 50 μl reconstitution buffer containing GTP γ^{35}S (final concentration, 7 nM). The ligand R-N^6-phenylisopropyladenosine (final concentration, 100 nM) was added 8 min later (t = 0). The reaction was stopped by removing membrane aliquots for filtration through nitrocellulose filters (HAWP-025; Millipore, Billerica, MA) at t = 2, 4, 6, and 7.5 min. The wash buffer consisted of 10 mM Tris and 5 mM $MgCl_2$ (pH 7.4). Radioactivity trapped on filters was measured using a scintillation counter (Beckman LS6500).[44]

Analysis

Results are reported as mean ± SEM. Student *t* test was used to determine statistically significant differences between paired groups, with $P < 0.05$ considered significant. Concentration–response curves were fitted to the following function, derived from the Hill equation: $y = y_{min} + (y_{max} - y_{min})[1 - x^n/(x_{50}{}^n + x^n)]$, in which y_{max} and y_{min} are the maximal and minimal response obtained; n is the Hill coefficient; and x_{50} is the half-maximal effect concentration (*i.e.*, EC_{50} for an agonist and IC_{50} for an antagonist). The slopes of the agonist-induced GTPγS binding were calculated by using linear regression (Prism 3.0).

Materials

Molecular biology reagents were purchased from Promega (Madison, WI), and other chemical reagents were obtained from Sigma (St. Louis, MO). Cell culture reagents were from Gibco Life Technologies, Inc. (Grand Island, NY), and radioligands were from NEN Life Sciences Products (Boston, MA). Stock solutions of lidocaine hydrochloride were freshly prepared in the required assay buffer and then diluted to the final concentrations.

Results

Nontransfected CHO cells lack endogenous adenosine receptors as determined by two criteria. First, A1R binding in control cells was indistinguishable from nonspecific binding (data not shown). Second, no A1R-mediated inhibition of adenylate cyclase was observed in control cells (data not shown).

Expression of hA1R in CHO Cells

To use CHO cells stably expressing hA1R as a suitable *in vitro* test system for LA effects on adenosine signaling,

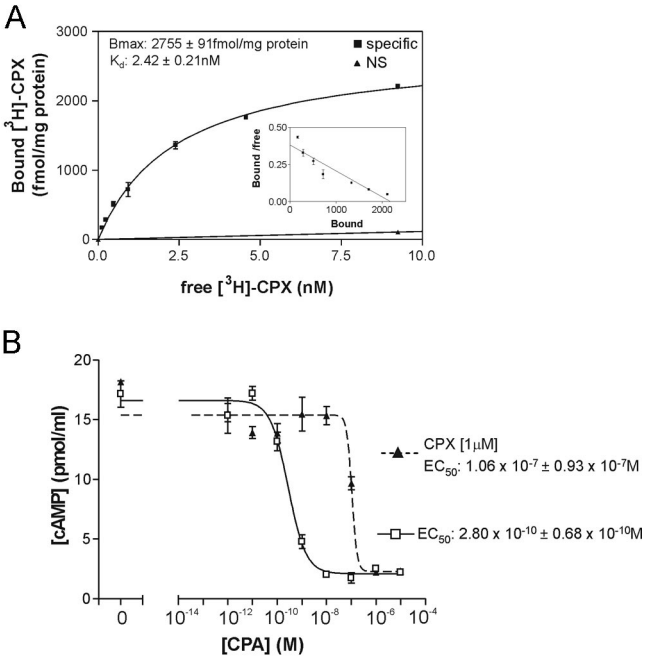

Fig. 2. (*A*) Quantification of human adenosine 1 receptors by characterization of [^{3}H]-8-cyclopentylxanthine (CPX) binding to membranes prepared from transfected Chinese hamster ovary cells (20 μg protein/tube). Saturation isotherm and Scatchard plot (*inset*) conform to a single-site model with a dissociation constant, K_d, of 2.42 ± 0.21 nM and a B_{max} of 2,755 ± 91 fmol/mg protein. Nonspecific (NS) binding was measured in the presence of excess 8-cyclopentyltheophylline (100 μM). Each *point* is the mean of triplicate determinations. Bound units for the Scatchard plot are expressed as fmol per assay tube. (*B*) Human adenosine 1 receptors expressed in Chinese hamster ovary cells were functionally active, thus decreasing cAMP levels on binding of the agonist N^6-cyclopentyladenosine (CPA) (10^{-12}–10^{-5} M). In agreement with the action of a competitive antagonist, addition of CPX (1 μM) resulted in a right shift of the dose–response curve, increasing EC_{50} values nearly 1,000-fold.

two requirements were necessary: A1Rs had to be expressed in sufficient numbers, and they had to be functional. We first determined the density of hA1R expressed in transfected CHO cells by radioligand binding studies using the specific antagonist [^{3}H]-CPX. In a range from 0.1 to 10 nM, free drug specific binding was saturable. The saturation curve and Scatchard analysis (fig. 2A) conform closely to a single-site model with a dissociation constant K_d of 2.42 ± 0.21 nM and a B_{max} of 2,755 ± 91 fmol/mg protein (n = 5), which confirmed sufficient expression. We then addressed the question whether transfected hA1 receptors were functionally active. If so, binding of an agonist should result in a decrease in cAMP (fig. 1). As shown in figure 2B, N^6-cyclopentyladenosine (CPA) (10^{-12}–10^{-5} M), an hA1R agonist, decreased cAMP formation in a concentration-dependent manner. Fitting to the Hill equation yielded an EC_{50} of $2.8 \times 10^{-10} \pm 0.68 \times 10^{-10}$ M. Maximal cAMP reduction by 88.0 ± 1.36% of baseline was obtained with a concentration of 1×10^{-6} M. Next we showed that addition of CPX (1 μM), a competitive antagonist, shifts the concentration response curve signif-

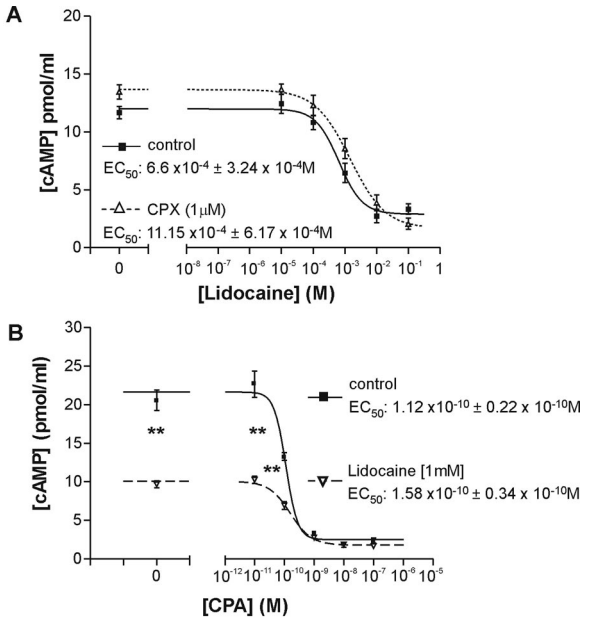

Fig. 3. Lidocaine enhances human adenosine 1 receptor–coupled signaling. (*A*) Lidocaine (10^{-5}–10^{-1} M) reduced cAMP in a concentration-dependent manner in the absence (ctrl) and in the presence of the adenosine 1 receptor antagonist 8-cyclopentylxanthine (CPX) at similar EC_{50}s. (*B*) This inhibitory effect was independent of the presence of an agonist: 1 mM lidocaine reduced cAMP by 47.6 ± 8.42% (lidocaine *vs.* control, $^{**}P < 0.001$) at a concentration of N^6-cyclopentyladenosine (CPA) of 10^{-10} M and by 52.1 ± 5.96% (lidocaine *vs.* control, $^{**}P < 0.001$) in the absence of the agonist CPA.

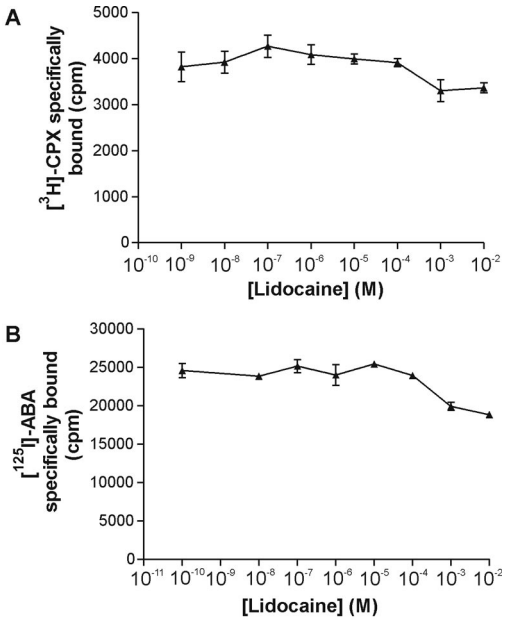

Fig. 4. Lidocaine in increasing concentrations did not significantly affect antagonist-specific binding (*A*) or agonist-specific binding (*B*), making the ligand binding pocket as the target site for the local anesthetic unlikely. [^{3}H]-CPX = [^{3}H]-8-cyclopentylxanthine; [^{125}I]-ABA = [^{125}I]-N^6-aminobenzyladenosine.

icantly to the right. EC_{50} for CPA in the presence of CPX was almost 1,000-fold higher ($1.06 \times 10^{-7} \pm 0.83 \times 10^{-7}$ M) (fig. 2B). Thus, CHO cells possess the appropriate $G\alpha_i$ protein to couple to the hA1R and use adenylate cyclase as an effector. These results were in accordance with previous findings.[29–32,35]

Lidocaine Enhances Adenosine 1 Receptor Signaling

To determine whether lidocaine had any effect on the entire A1R-coupled signaling pathway, we used cAMP assays. Even in the absence of an agonist, lidocaine (10^{-5}–10^{-1} M) reduced cAMP levels in a concentration-dependent manner with a calculated EC_{50} of $6.60 \times 10^{-4} \pm 3.24 \times 10^{-4}$ M (fig. 3A). Possible explanations of this effect may be that lidocaine acts as an agonist at the ligand binding site of the hA1 receptor or that it activates the pathway further downstream at the receptor itself or somewhere within the signaling cascade.

Lidocaine Is Not an hA1R Agonist

To characterize the underlying mechanism by which lidocaine enhanced hA1R-coupled signaling, we studied the inhibition of cAMP production by increasing concentrations of the hA1R agonist CPA (10^{-11}–10^{-7} M) in the absence and presence of lidocaine at approximately EC_{50} (1 mM) (fig. 3B). Lidocaine significantly reduced cAMP production to approximately 50% of baseline ($P < 0.001$) in the absence of CPA and in the presence of CPA (10^{-11}–10^{-10} M). Under control conditions (in the absence of lidocaine), cAMP concentrations range from around 10–20 pmol/ml at CPA concentrations from 10^{-11}–10^{-10} M, and they were reduced significantly to 5–10 pmol/ml in the presence of lidocaine (at the same CPA concentrations). This shows the additional effect of lidocaine on cAMP reduction and therefore its ability to potentiate A1R signaling. At CPA concentrations greater than 10^{-9} M, no additional effect of lidocaine was obtained, most likely because of the already low level of cAMP formation. The calculated EC_{50}'s for CPA ($1.12 \times 10^{-10} \pm 0.22 \times 10^{-10}$ M in the absence of lidocaine and $1.58 \times 10^{-10} \pm 0.34 \times 10^{-10}$ M in the presence of lidocaine) did not differ significantly. In addition, lidocaine maintained its inhibitory effect on cAMP formation even in the presence of the A1R antagonist CPX, and at similar EC_{50} ($11.15 \times 10^{-4} \pm 6.17 \times 10^{-4}$ M) (fig. 3A). Together, these findings suggest a site of action for lidocaine separate from the agonist binding site.

We confirmed this finding using binding studies. Because agonist and antagonist binding kinetics differ, we measured binding of the antagonist [^{3}H]-CPX or the agonist [^{125}I]-ABA at their dissociation constant K_d, in the presence of increasing concentrations of lidocaine (10^{-10}–10^{-2} M). Consistent with the functional data, lidocaine affected neither antagonist (fig. 4A) nor agonist (fig. 4B) binding to the hA1R significantly. At the functionally determined EC_{50} ($6.60 \times 10^{-4} \pm 3.24 \times 10^{-4}$ M), only insignificant inhibition of binding occurred,

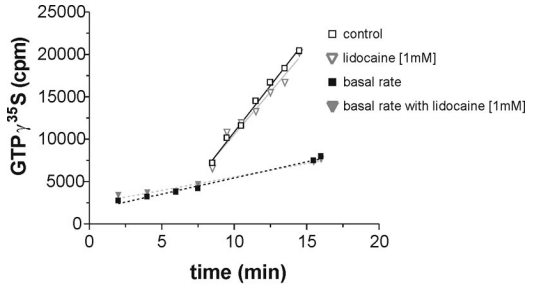

Fig. 5. GDP/GTP γ^{35}S exchange assays using human adenosine 1 receptors reconstituted with α_{i1}(20 nM)/$\beta_{1\gamma2}$(40 nM). Binding of the adenosine 1 receptor agonist R-N^6-phenylisopropyladenosine stimulates [^{35}S]GTP γS binding to Gα_i and shows the ability of reconstituted Gα_{i1}/$\beta_{1\gamma2}$ to couple to the human adenosine 1 receptor (*open squares*). Lidocaine (1 mM) was without effect on receptor G protein interactions (*open triangles*), because both slopes did not differ significantly. Agonist-stimulated GTP γ^{35}S binding and basal rates were determined using linear regression. The data shown represent the average of three experiments. GDP = guanosine diphosphate; GTP γ^{35}S = γ^{35}S guanosine triphosphate.

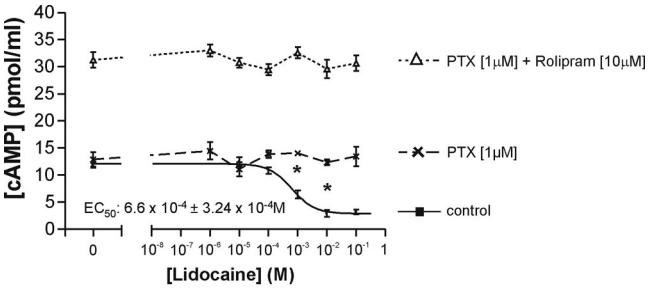

Fig. 6. Lidocaine reduces cAMP production in a concentration-dependent manner (*squares*). This effect was completely abolished by preincubation of Chinese hamster ovary cells with the Gα_i inhibitor pertussis toxin (PTX) (*X's*), (control *vs.* PTX [1 μM], *$P < 0.001$). Addition of the phospodiesterase inhibitor rolipram resulted, as expected, in an increase of cAMP because of its reduced breakdown. However, rolipram did not have any effects on the cAMP-reducing effects of lidocaine nor on the effects of PTX (*triangles*).

which cannot explain the highly significant reduction of the cAMP levels. If anything, activation of hA1R signaling at the receptor level should have become evident in an increased binding of an agonist to the receptor rather than a decreased binding. These results make an interaction of lidocaine with the agonist–antagonist binding site highly unlikely. The site of action for lidocaine within the Gα_i-coupled signaling pathway therefore must be somewhere else at the receptor level or further downstream in the signaling cascade.

Lidocaine Does Not Interfere with Receptor–G Protein Coupling, GDP/GTP Exchange, or Gα_i/$\beta_{1\gamma2}$ Subunit Interaction

We next assessed lidocaine interference with A1R coupling to the Gα_i protein, GDP/GTP exchange at the Gα_i subunit, and interaction between Gα_i and the $\beta_{1\gamma2}$ dimers. For these experiments, the hA1R was reconstituted with pure Gα_{i1} and $\beta_{1\gamma2}$ subunits, and agonist-stimulated GTP γ^{35}S binding was measured as described in the Materials and Methods section. The data illustrated in figure 5 support the ability of Gα_{i1} and $\beta_{1\gamma2}$ subunits to couple to the hA1 receptor, as indicated by the marked change in the rate of GTP γ^{35}S bound after addition of 100 nM of the specific A1R agonist R-N^6-phenylisopropyladenosine. Because lidocaine (1 mM) did not significantly alter the slopes of the curves of unstimulated or R-N^6-phenylisopropyladenosine–stimulated GTP γS binding (fig. 5), we concluded that LAs do not affect receptor–Gα_i protein coupling, GDP/GTP exchange, or the interaction between Gα_i and $\beta_{1\gamma2}$ subunits.

Lidocaine Does Not Stimulate Phosphodiesterase

By adding the phosphodiesterase inhibitor rolipram (10 μM) to cAMP assays, we examined whether the significant reduction of cAMP levels by lidocaine was because of a stimulating effect on phosphodiesterase, which would lead to an accelerated breakdown of cAMP. As expected, cAMP levels were increased in the presence of the phosphodiesterase inhibitor. However, rolipram had no effect on the lidocaine-induced cAMP reduction, leaving its concentration–response curve (fig. 3A) and the EC$_{50}$ (data not shown) unaffected. If lidocaine's cAMP reducing effects would have been caused completely or partly by stimulation of phosphodiesterase, this effect should have been abolished or reduced after the addition of rolipram. Thus, we can exclude phosphodiesterases, or at least those inhibited by the phosphodiesterase inhibitor rolipram, as a primary site of action for lidocaine.

Lidocaine-induced Decrease in cAMP Levels Is PTX-sensitive

In contrast, inhibition of cAMP formation by lidocaine was completely abolished after 24 h incubation of transfected CHO cells with PTX (1 μM). PTX inactivates the Gα_i protein and excludes a site of action for lidocaine downstream of the G protein in the signaling pathway. Again, the addition of rolipram only increased cAMP levels, but did not have any additional effect on the action of lidocaine (fig. 6). These data therefore indicate that stimulation of Gα_i function is the most likely cause of lidocaine-induced decreases in cAMP levels.

Discussion

As part of our program investigating LA interactions with G protein-coupled receptor signaling, we investigated lidocaine effects on Gα_i protein function by using the hA1R signaling cascade as a model. In contrast to previous studies showing inhibitory actions of LAs on Gα_q, lidocaine potentiates the hA1R signaling cascade, most likely by enhancing Gα_i protein function. Lidocaine did not significantly interact with the ligand binding site

nor did the LAs affect the signaling pathway downstream of the $G\alpha_i$ protein.

Agonist and antagonist binding were inhibited only marginally by high concentrations of lidocaine, making the ligand binding pocket as target site for the LAs unlikely. Nevertheless, the receptor as an additional target site for LA action cannot be excluded completely because lidocaine may bind to extracellular receptor domains other than agonist and antagonist binding sites and hence allosterically activate A1R signaling. However, one would expect this to result in altered receptor–G protein coupling. Because lidocaine did not have any effect on receptor–G protein interaction, as shown by the GDP/GTP exchange assays, this possibility appears less likely.

It is noteworthy that enhancement of $G\alpha_i$ function did not require the presence of an agonist (fig. 3). Endogenous adenosine cannot have acted as an agonist, because all endogenous CHO adenosine should have been inactivated by ADA. Lidocaine even maintained its cAMP-reducing effect in the presence of an A1R antagonist (fig. 3B). Taken together, these findings suggest that LA action does not require the A1R. This hypothesis is supported by a study by Hirota *et al.*,[34] who found that lidocaine produced a concentration-dependent and naloxone-insensitive inhibition of cAMP formation in nontransfected CHO cells. The EC_{50} was 2.78 mM (1.63–4.73 mM), similar to the EC_{50} found by us (660 μM–1.6 mM) (fig. 3A). However, Hirota *et al.* did not investigate the mechanism underlying this finding. Our results support the hypothesis that lidocaine interacts directly with the $G\alpha_i$ protein, thereby leading to reduced cAMP formation. As to the exact mechanism by which LAs enhance $G\alpha_i$ function, several possible explanations can be ruled out by the GDP/GTP exchange assay results: First, LAs did not interfere with receptor–G protein coupling, neither by increasing the affinity of the G protein for the receptor, nor by enhancing uncoupling of the G protein from the receptor. Second, LAs did not affect GDP/GTP exchange at the $G\alpha_i$ subunit. A direct activation of GDP-bound $G\alpha_i$ by lidocaine, for which the GDP/GTP exchange would be a prerequisite, can therefore essentially be excluded or is highly unlikely. Third, LAs did not affect interaction between $G\alpha_i$ and the $\beta_{1\gamma 2}$ dimers. Instead, the results suggest that lidocaine interacts with the pool of already activated $G\alpha_i$ present in the cytoplasm and thereby facilitates its ability to inhibit adenylate cyclase. At least two potential explanations for this facilitation of $G\alpha_i$ activity may exist. First, LAs might inhibit $G\alpha_i$-related GTPase activity. Inhibition of GTPase activity could be a direct effect of LAs or could be the result of inhibitory action of LA on GTPase-activating proteins. Second, LAs might stabilize the $G\alpha_i$–adenylate cyclase complex. Both mechanisms would prolong the half-life of the activated $G\alpha_i$–GTP complex, resulting in reduced cAMP formation. Future experiments in isolated systems such as phospholipid vesicles, allowing reconstitution of the separate components being involved, including the various adenylate cyclase isoforms, will be necessary to define more precisely the interaction sites and the exact molecular mechanism. A direct inhibitory effect of lidocaine on adenylate cyclase can be excluded because PTX completely abolished the effect of lidocaine. The activated $G\alpha_i$ present in the cytoplasm, even in the absence of an agonist, could derive from at least two sources. First, G protein–coupled receptors have classically been viewed to have an inactive conformation (R) that requires an agonist-induced conformational change for receptor–G protein coupling and, thus, G protein activation to occur. New evidence suggests, however, a more complex two (or more)-state model, in which receptors are in equilibrium between the inactive conformation (R) and a spontaneously active state (R*). Classic agonists serve as catalysts and increase the concentration of R*. An equilibrium between R and R*, however, implies that at any time a certain proportion of receptors are in the R* state that can couple to the G protein, even in the absence of a ligand, thus resulting in release of $G\alpha_I$–GTP into the cytosol. Second, GDP/GTP exchange at the $G\alpha_i$ subunit occurs to some extent, even in the absence of receptor stimulation. This spontaneous GDP release is reflected in our GDP/GTP γS exchange assays as the basal rate (fig. 5).[45,46]

Potentiation of A1 adenosine signaling by lidocaine independent of an agonist and receptor inevitably raises two questions: Does lidocaine also enhance hA1R signaling with an effector other than adenylate cyclase? Are other $G\alpha_i$-coupled receptor systems affected in the same way? A1Rs were originally characterized on the basis of their ability to inhibit adenylate cyclase *via* the $G\alpha_i$ protein (fig. 1). Meanwhile, a number of other PTX-sensitive effector mechanisms of A1Rs have been discovered, including increases in K^+-channel conductance, decreases in Ca^{2+}-channel conductance, and increases or decreases in phospholipase C activity.[33,39] Testing the effects of LA on these effector systems would help to further clarify the mechanism by which LAs enhance $G\alpha_i$ function and whether it depends on cAMP. If further studies show that these effectors are also affected by lidocaine in a PTX-sensitive, agonist-independent manner, this would tremendously increase the number of potentially clinically relevant signaling pathways that are affected by LAs. A great number of receptors signal *via* $G\alpha_i$ proteins (*e.g.*, α_2-adrenergic, dopaminergic (D2, D3, D4), m2 and m4 muscarinic, $GABA_B$, and metabotropic glutamate receptors).[47,48] If lidocaine affects other $G\alpha_i$-coupled signaling cascades in the same way as A1 signaling, a number of relevant pathways may be affected.

We used lidocaine as a prototypical compound; it is conceivable that other compounds might yield different results. We also realize that our findings and those of others[34] might be biased by the *in vitro* test system used

(CHO cells). However, lidocaine and other LAs (*e.g.*, bupivacaine, mepivacaine, and ropivacaine) have all been found (albeit with varying potencies) to reduce cAMP levels in more physiologic preparations, such as cardiomyocytes,[13,49] erythrocytes,[50] and lymphocytes.[12,51] However, the underlying molecular mechanisms were not investigated in detail or remained unclear. Some of the studies suggested the receptor as the target, others the adenylate cyclase system. For example, Roux *et al.*[13] reported that the inhibitory effect of lidocaine (up to 10 mM) on isoproterenol- and forskolin-stimulated cAMP was reduced by 25% in the presence of cholera toxin, but it was completely abolished in the presence of PTX. The authors hypothesized that "lidocaine's interaction with the adenylate cyclase system" could contribute to the antiarrhythmic and the negative inotropic effects *in vivo*. However the mechanism underlying this interaction with the adenylate cyclase system was not further investigated. This issue may partly be associated with the heterogeneous background of these native cell preparations; for example, cAMP reduction might be the result of inhibition of $G\alpha_s$ or enhancement of $G\alpha_i$-coupled signaling. Nevertheless, these observations in other models make it unlikely that our results simply represent an artifact of the transfection system or a unique property of lidocaine. In any case, additional experiments in various other physiologic preparations will be necessary to further define our findings and to test whether the data obtained with lidocaine can be extrapolated to other LAs and to other tissues.

The LA concentrations required to affect G protein function may appear to be relatively high. However, it is important to keep in mind that the concentrations of LA used in clinical practice vary widely depending on the method of application.[52] After intravenous administration and similarly after epidural anesthesia, plasma concentrations in the low micromolar range are obtained.[53,54] In contrast, LA concentrations routinely observed after topical application or tissue infiltration are approximately 1,000-fold greater. Similarly, millimolar LA concentrations are also present around the spinal nerves after spinal or epidural administration of an LA.[1,2,55,56] Therefore, concentrations tested in our study are well within the range of those obtained *in vivo*. Interestingly, most *in vitro* studies require much greater LA concentrations than *in vivo* studies, in which the same or similar phenomenon often occurs at much lower concentrations.[2] Although not fully understood, one reason for this discrepancy may well be that the multitude of molecular targets present in a complex *in vivo* system allows interactions of diverse LA effects, which cannot be attained in a simplified *in vitro* system. Thus, the previously mentioned cAMP-reducing effects of various LAs in cardiomyocytes[13,49] or lymphocytes[12,51] may represent the result of blockade of β-adrenergic receptors, and enhancement of $G\alpha_i$ function and even inhibition of $G\alpha_s$.

Adenosine administered systemically or injected spinally has been shown to exert long-lasting and significant antinociception under conditions of neuropathic pain, with no effect on acute pain.[57,58] By using spinal nerve ligation as a model for neuropathic pain, it was reported that adenosine release in the spinal cord is stimulated and results in increased G protein activity.[59] Our findings that lidocaine facilitates $G\alpha_i$ function thus may in part provide the molecular basis for the clinical application of lidocaine therapy in chronic pain states associated with hyperalgesia and allodynia.[60] The combination of increased adenosine levels in addition to lidocaine's action might result in cAMP concentrations sufficiently low to provide antinociception.

In summary, our study shows that lidocaine interacts with hA1R-coupled signaling, most likely by facilitating $G\alpha_i$ protein function. Expanding on previous findings, our data suggest that LA action depends on the specific G protein subunit affected and further support the hypothesis that specific G proteins represent an alternative target site of LA, separate from their well known actions on the Na^+-channel.

The authors thank Heidi Figler, B.S. (Department of Internal Medicine, University of Virginia, Charlottesville, Virginia), for her excellent technical assistance and Dr. Norbert Roewer (Department of Anesthesiology, University of Wuerzburg, Wuerzburg, Germany) for his support.

References

1. Butterworth JF, Strichartz GR: Molecular mechanisms of local anesthesia: A review. ANESTHESIOLOGY 1990; 72:711–34
2. Hollmann MW, Durieux ME: Local anesthetics and the inflammatory response: A new therapeutic indication? ANESTHESIOLOGY 2000; 93:858–75
3. Hollmann MW, Durieux ME: Local anesthetics: Effects on inflammation, wound healing and coagulation. Progress in Anesthesiology 2000; 14:291–304
4. Hollmann MW, Durieux ME: Local anesthetics: Effects on the central nervous system and bronchial reactivity. Progress in Anesthesiology 2000; 14: 323–36
5. Bjorck S, Dahlstrom A, Ahlman H: Treatment of distal colitis with local anaesthetic agents. Pharmacol Toxicol 2002; 90:173–80
6. Groudine SB, Fisher HA, Kaufman RP Jr, Patel MK, Wilkins LJ, Mehta SA, Lumb PD: Intravenous lidocaine speeds the return of bowel function, decreases postoperative pain, and shortens hospital stay in patients undergoing radical retropubic prostatectomy. Anesth Analg 1998; 86:235–9
7. Rimback G, Cassuto J, Tollesson PO: Treatment of postoperative paralytic ileus by intravenous lidocaine infusion. Anesth Analg 1990; 70:414–9
8. Rimback G, Cassuto J, Faxen A, Hogstrom S, Wallin G, Tollesson PO: Effect of intra-abdominal bupivacaine instillation on postoperative colonic motility. Gut 1986; 27:170–5
9. Hollmann MW, Wieczorek KS, Smart M, Durieux ME: Epidural anesthesia prevents hypercoagulation in patients undergoing major orthopedic surgery. Reg Anesth Pain Med 2001; 26:215–22
10. Hahnenkamp K, Theilmeier G, Van Aken HK, Hoenemann CW: The effects of local anesthetics on perioperative coagulation, inflammation, and microcirculation. Anesth Analg 2002; 94:1441–7
11. Tuman KJ, McCarthy RJ, March RJ, DeLaria GA, Patel RV, Ivankovich AD: Effects of epidural anesthesia and analgesia on coagulation and outcome after major vascular surgery. Anesth Analg 1991; 73:696–704
12. Butterworth JF, Brownlow RC, Leith JP, Prielipp RC, Cole LR: Bupivacaine inhibits cyclic-3', 5'-adenosine monophosphate production: A possible contributing factor to cardiovascular toxicity. ANESTHESIOLOGY 1993; 79:88–95
13. Roux S, Escoubet B, Friedlander G, Le Grimellec C, Bertrand I, Amiel C: Effects of lidocaine on sarcolemmal fluidity and cellular cAMP in rat cardiomyocytes. Am J Physiol 1989; 256:H422–7
14. Xiong Z, Bukusoglu C, Strichartz GR: Local anesthetics inhibit the G

protein-mediated modulation of K+ and Ca++ currents in anterior pituitary cells. Mol Pharmacol 1999; 55:150–8

15. Lipka LJ, Jiang M, Tseng GN: Differential effects of bupivacaine on cardiac K channels: Role of channel inactivation and subunit composition in drug-channel interaction. J Cardiovasc Electrophysiol 1998; 9:727–42

16. Valenzuela C, Delpon E, Tamkun MM, Tamargo J, Snyders DJ: Stereoselective block of a human cardiac potassium channel (Kv1.5) by bupivacaine enantiomers. Biophys J 1995; 69:418–27

17. Rossner KL, Freese KJ: Bupivacaine inhibition of L-type calcium current in ventricular cardiomyocytes of hamster. ANESTHESIOLOGY 1997; 87:926–34

18. Nietgen GW, Chan CK, Durieux ME: Inhibition of lysophosphatidate signaling by lidocaine and bupivacaine. ANESTHESIOLOGY 1997; 86:1112–9

19. Sullivan LM, Honemann CW, Arledge JA, Durieux ME: Synergistic inhibition of lysophosphatidic acid signaling by charged and uncharged local anesthetics. Anesth Analg 1999; 88:1117–24

20. Fischer LG, Bremer M, Coleman EJ, Conrad B, Krumm B, Gross A, Hollmann MW, Mandell G, Durieux ME: Local anesthetics attenuate lysophosphatidic acid-induced priming in human neutrophils. Anesth Analg 2001; 92:1041–7

21. Honemann CW, Arledge JA, Podranski T, Aken HV, Durieux M: Volatile and local anesthetics interfere with thromboxane A2 receptors recombinantly expressed in *Xenopus* oocytes. Adv Exp Med Biol 1999; 469:277–83

22. Honemann CW, Lo B, Erera JS, Polanowska-Grabowska R, Gear AR, Durieux M: Local anesthetic effects on TXA2 receptor mediated platelet aggregation using quenched flow aggregometry. Adv Exp Med Biol 1999; 469:269–76

23. Hollmann MW, Gross A, Jelacin N, Durieux ME: Local anesthetic effects on priming and activation of human neutrophils. ANESTHESIOLOGY 2001; 95:113–22

24. Honemann CW, Heyse TJ, Mollhoff T, Hahnenkamp K, Berning S, Hinder F, Linck B, Schmitz W, van Aken H: The inhibitory effect of bupivacaine on prostaglandin E(2) (EP(1)) receptor functioning: Mechanism of action. Anesth Analg 2001; 93:628–34

25. Hollmann MW, Fischer LG, Byford AM, Durieux ME: Local anesthetic inhibition of m1 muscarinic acetylcholine signaling. ANESTHESIOLOGY 2000; 93: 497–509

26. Hollmann MW, Ritter CH, Henle P, de Klaver M, Kamatchi GL, Durieux ME: Inhibition of m3 muscarinic acetylcholine receptors by local anaesthetics. Br J Pharmacol 2001; 133:207–16

27. Hollmann MW, Wieczorek KS, Berger A, Durieux ME: Local anesthetic inhibition of G protein-coupled receptor signaling by interference with Galpha(q) protein function. Mol Pharmacol 2001; 59:294–301

28. Aronstam RS: G protein systems, Anesthesia: Biologic Foundations, 1st edition. Edited by Yaksh TL, Lynch C, Zapol WM, Maze M, Biebuyck JF, Saidman LJ. Philadelphia, Lippincott-Raven, 1997, pp 45–53

29. Freund S, Ungerer M, Lohse MJ: A1 adenosine receptors expressed in CHO-cells couple to adenylyl cyclase and to phospholipase C. Naunyn Schmiedebergs Arch Pharmacol 1994; 350:49–56

30. Linden J, Auchampach JA, Jin X, Figler RA: The structure and function of A1 and A2B adenosine receptors. Life Sci 1998; 62:1519–24

31. Gao Z, Robeva AS, Linden J: Purification of A1 adenosine receptor-G-protein complexes: Effects of receptor down-regulation and phosphorylation on coupling. Biochem J 1999; 338(pt 3):729–36

32. Robeva AS, Woodard R, Jin X, Gao Z, Bhattacharya S, Taylor HE, Rosin DL, Linden J: Molecular characterization of recombinant human adenosine receptors. Drug Dev Res 1996; 39:243–52

33. Dickenson JM, Hill SJ: Involvement of G-protein betagamma subunits in coupling the adenosine A1 receptor to phospholipase C in transfected CHO cells. Eur J Pharmacol 1998; 355:85–93

34. Hirota K, Okawa H, Appadu BL, Grandy DK, Lambert DG: Interaction of local anaesthetics with recombinant mu, kappa, and delta-opioid receptors expressed in Chinese hamster ovary cells. Br J Anaesth 2000; 85:740–6

35. Klotz KN, Hessling J, Hegler J, Owman C, Kull B, Fredholm BB, Lohse MJ: Comparative pharmacology of human adenosine receptor subtypes: Characterization of stably transfected receptors in CHO cells. Naunyn Schmiedebergs Arch Pharmacol 1998; 357:1–9

36. Graham S, Combes P, Crumiere M, Klotz KN, Dickenson JM: Regulation of p42/p44 mitogen-activated protein kinase by the human adenosine A3 receptor in transfected CHO cells. Eur J Pharmacol 2001; 420:19–26

37. Schulte G, Fredholm BB: Human adenosine A(1), A(2A), A(2B), and A(3) receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2. Mol Pharmacol 2000; 58:477–82

38. Linden J: Structure and function of A1 adenosine receptors. FASEB J 1991; 5:2668–76

39. Linden J: Purinergic systems, Basic Neurochemistry: Molecular, Cellular, and Medical Aspects, 5th edition. Edited by Siegel GJ, Agranoff BW, Albers RW, Molinoff PB. New York, Raven Press, 1994, pp 401–16

40. Robeva AS, Woodard R, Luthin DR, Taylor HE, Linden J: Double tagging recombinant A1- and A2-adenosine receptors with hexahistidine and the FLAG epitope: Development of an efficient generic protein purification procedure. Biochem Pharmacol 1996; 51:545–55

41. Harper JF, Brooker G: Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'0 acetylation by acetic anhydride in aqueous solution. J Cyclic Nucleotide Res 1975; 1:207–18

42. Graber SG, Figler RA, Garrison JC: Expression and purification of functional G protein alpha subunits using a baculovirus expression system. J Biol Chem 1992; 267:1271–8

43. Graber SG, Figler RA, Kalman-Maltese VK, Robishaw JD, Garrison JC: Expression of functional G protein beta gamma dimers of defined subunit composition using a baculovirus expression system. J Biol Chem 1992; 267: 13123–6

44. McIntire WE, Myung CS, MacCleery G, Wang Q, Garrison JC: Reconstitution of G protein-coupled receptors with recombinant G protein alpha and beta gamma subunits. Methods Enzymol 2002; 343:372–93

45. Bond RA, Leff P, Johnson TD, Milano CA, Rockman HA, McMinn TR, Apparsundaram S, Hyek MF, Kenakin TP, Allen LF: Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the beta 2-adrenoceptor. Nature 1995; 374:272–6

46. Gudermann T, Kalkbrenner F, Schultz G: Diversity and selectivity of receptor-G protein interaction. Annu Rev Pharmacol Toxicol 1996; 36:429–59

47. Maze M, Buttermann A: G protein-coupled receptors, Anesthesia: Biologic Foundations, 1st edition. Edited by Yaksh TL, Lynch C, Zapol WM, Maze M, Biebuyck JF, Saidman LJ. Philadelphia, Lippincott-Raven, 1997, pp 55–64

48. Yost CS: G proteins: Basic characteristics and clinical potential for the practice of anesthesia. Anesth Analg 1993; 77:822–34

49. Illiano G, Chiosi E, Draetta GF, Laurenza A: Relationship between the fluidity of the membrane lipids and the activity of the membrane-bound proteins: The effects of lidocaine on the adenylate cyclase activity of rat myocardium. Gen Pharmacol 1983; 14:669–72

50. Voeikov VL, Lefkowitz RJ: Effects of local anesthetics on guanyl nucleotide modulation of the catecholamine-sensitive adenylate cyclase system and on beta-adrenergic receptors. Biochim Biophys Acta 1980; 629:266–81

51. Butterworth J, James RL, Grimes J: Structure-affinity relationships and stereospecificity of several homologous series of local anesthetics for the beta2-adrenergic receptor. Anesth Analg 1997; 85:336–42

52. Eckenhoff RG, Johansson JS: On the relevance of "clinically relevant concentrations" of inhaled anesthetics in *in vitro* experiments. ANESTHESIOLOGY 1999; 91:856–60

53. Collinsworth KA, Kalman SM, Harrison DC: The clinical pharmacology of lidocaine as an antiarrhythmic drug. Circulation 1974; 50:1217–30

54. Mayumi T, Dohi S, Takahashi T: Plasma concentrations of lidocaine associated with cervical, thoracic, and lumbar epidural anesthesia. Anesth Analg 1983; 62:578–80

55. Holst D, Mollmann M, Scheuch E, Meissner K, Wendt M: Intrathecal local anesthetic distribution with the new spinocath catheter. Reg Anesth Pain Med 1998; 23:463–8

56. Covino BG, Scott DB, Lambert DH: Handbook of Spinal Anesthesia and Analgesia. Philadelphia, WB Saunders, 1994, pp 1–168

57. Pan HL, Xu Z, Leung E, Eisenach JC: Allosteric adenosine modulation to reduce allodynia. ANESTHESIOLOGY 2001; 95:416–20

58. Sjolund KF, Belfrage M, Karlsten R, Segerdahl M, Arner S, Gordh T, Solevi A: Systemic adenosine infusion reduces the area of tactile allodynia in neuropathic pain following peripheral nerve injury: A multi-centre, placebo-controlled study. Eur J Pain 2001; 5:199–207

59. Bantel C, Childers SR, Eisenach JC: Role of adenosine receptors in spinal G-protein activation after peripheral nerve injury. ANESTHESIOLOGY 2002; 96:1443–9

60. Mao J, Chen LL: Systemic lidocaine for neuropathic pain relief. Pain 2000; 87:7–17