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# *Influence of Volatile Anesthetics on Thromboxane $A_2$ Signaling*

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***Background:*** **Thromboxane $A_2$ ($TXA_2$) is a member of the prostaglandin family; activation of its receptor induces several important effects, including platelet aggregation and smooth muscle contraction. Because volatile anesthetics interfere with aggregation and contraction, the authors investigated effects of halothane, isoflurane, and sevoflurane on $TXA_2$ signaling in an isolated receptor model.**

***Methods:*** **mRNA encoding $TXA_2$ receptors was prepared *in vitro* and expressed in *Xenopus* oocytes. The effects of halothane, isoflurane, and sevoflurane on $Ca_{2+}$-activated $Cl^-$ currents induced by the $TXA_2$ agonist U-46619 and on those induced by intracellular injection of inositol 1-4-5 trisphosphate or guanosine 5′-O-(2-thiodiphosphate) were measured using the voltage-clamp technique.**

***Results:*** **Expressed $TXA_2$ receptors were functional (half maximal effect concentration [$EC_{50}$], $3.2 \times 10^{-7} \pm 1.1 \times 10^{-7}$ M; Hill coefficient (h), 0.8 ± 0.2). Halothane and isoflurane inhibition of $TXA_2$ signaling was reversible and concentration dependent (halothane half maximal inhibitory concentration [$IC_{50}$], 0.46 ± 0.04 mM; h, 1.6 ± 0.21; isoflurane $IC_{50}$, 0.69 ± 0.12 mM; h, 1.3 ± 0.27). 0.56 mM halothane (1%) right-shifted the U-46619 concentration-response relationship by two orders of magnitude ($EC_{50}$, $1 \times 10^{-5}$ M). That h and maximal effect ($E_{max}$) were unchanged indicates that halothane acts in a competitive manner. In contrast, isoflurane acted noncompetitively, decreasing $E_{max}$ by 30% (h and $EC_{50}$ were unchanged). Both halothane and isoflurane had no effect on intracellular signaling pathways. Sevoflurane (0–1.3 mM) did not affect $TXA_2$ signaling.**

***Conclusions:*** **Both halothane and isoflurane inhibit $TXA_2$ signaling at the membrane receptor, but by different mechanisms. This suggests that the effects of these anesthetics on $TXA_2$ signaling are evoked at different locations of the receptor protein: halothane probably acts at the ligand binding site and isoflurane at an allosteric site. (Key words: Allosteric; competitive; G protein-coupled receptor; halothane; inhibition; isoflurane; sevoflurane; signaling pathway.)**

VOLATILE anesthetics interact with various cellular systems. Whereas anesthetic-lipid interactions were studied in detail in past decades, more recent emphasis has focused on the interactions between anesthetics and membrane proteins. It has indeed been shown that such interactions exist and that these modulations may be important in bringing about the anesthetic state. Ligand-gated ion channels, such as gamma-aminobutyric acid[1] receptors, have received particular attention. However, interactions with other proteins also may be important, either in modulatory roles or by inducing anesthetic side effects.

The large superfamily of G protein-coupled membrane receptors is a group of proteins that has been studied in less detail, despite the fact that it contains many members of great relevance to anesthesiologists (such as muscarinic acetylcholine and adrenergic, opiate, and eicosanoid receptors). In addition, investigations of serotonin, muscarinic acetylcholine,[2] and lysophosphatidate[3] receptors have shown interactions between anesthetics and G protein-coupled receptors.

Receptors for lipid mediators are of particular interest

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in this regard, because a hydrophobic ligand binding domain might be a likely site of anesthetic action. Lipid mediators—such as prostanoids, leukotrienes, and platelet activating factor—are important intercellular messengers and have pronounced biologic effects (platelet aggregation, smooth muscle contraction, pain, and inflammation). These effects are induced by activating specific membrane receptors, resulting in increased intracellular $Ca^{2+}$ concentrations and changes in other second messenger systems.

Thromboxane $A_2$ ($TXA_2$) is a prominent member of the prostanoid family and has various actions on cell and tissue functions of particular interest to anesthesiologists. For example, activation of $TXA_2$ receptors induces platelet aggregation and vascular and bronchiolar smooth muscle contraction.[4,5] Because several anesthetics exhibit effects opposite to these, we hypothesized that $TXA_2$ receptor signaling could be a target for volatile anesthetics. If anesthetics indeed inhibit signaling by hydrophobic interactions at the ligand-binding pocket, we would expect that various anesthetics would have similar effects, with potencies related to their lipid solubility. The effects of halothane, isoflurane, and sevoflurane on $TXA_2$-induced platelet aggregation have been studied,[5,6] but results are contradictory and direct anesthetic effects on receptor functioning have not been investigated.

*Xenopus* oocytes form a flexible system to study recombinantly expressed G protein–coupled receptors and the influence of volatile anesthetics on their functioning.[7] Therefore, we expressed rat $TXA_2$ receptors in *Xenopus* oocytes and investigated the influence of halothane, isoflurane, and sevoflurane on their functioning and on the intracellular signaling pathways. The study was designed to answer the following questions:

1. Do halothane, isoflurane, and sevoflurane, at clinically relevant concentrations, modulate $TXA_2$ receptor signaling?
2. Are the anesthetic effects localized at the membrane receptor or within the intracellular signaling pathway?
3. Are there differences in effect between the anesthetics that could be related to different sites of action?

## Materials and Methods

### *Animals*

The study protocol was approved by the Animal Research Committee at the University of Virginia. Female *Xenopus laevis* toads were obtained from Xenopus I (Ann Arbor, MI), housed in an established frog colony, and fed regular frog brittle twice weekly. To remove the oocytes, a frog was anesthetized by immersion in 0.2% 3-amino-benzoic-methyl-ester until it was unresponsive to a painful stimulus (toe pinching). Animals were operated while positioned on ice. A 1-cm-long abdominal incision was made and a lobule of ovarian tissue, containing approximately 200 oocytes, was removed and placed in modified Barth's solution (containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 0.3 mM $Ca_2NO_3$, 0.1 mM gentamicin, and 15 mM HEPES, *p*H adjusted to 7.6). The wound was closed in two layers and the frog was allowed to recover from anesthesia. The oocytes were defolliculated by gentle shaking in a 1 mg/ml solution of collagenase type Ia in calcium-free OR2 solution (containing 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, *p*H adjusted to 7.5) for 2 h. After this process, the oocytes were returned to modified Barth's solution. Microscopic observation confirmed the absence of follicle cells.

### *mRNA Synthesis and Injection*

The rat $TXA_2$ receptor clone was obtained from Dr. K. R. Lynch (Department of Pharmacology, University of Virginia, Charlottesville, VA) as a cDNA encoding a 343 amino acid protein in the pcDNAI vector (Invitrogen, San Diego, CA). The construct was linearized with the nuclease *Xho* I and transcribed in the presence of capping analog by T7 polymerase, using a commercial RNA preparation kit (mMessage mMachine; Ambion, Austin, TX). Oocytes were injected with 5 ng mRNA in 30.6 nl sterile water, using an automated microinjector (Nanoject; Drummond Scientific, Broomall, PA). Microinjector functioning was confirmed by noting a slight increase in cell size during injection. The cells were then cultured in modified Barth's solution for 72 h before study.

### *Electrophysiologic Recording*

A single defolliculated oocyte was placed in a continuous-flow recording chamber (0.5 ml volume), perfused (3 ml/min) with Tyrode's solution (containing 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM dextrose, 10 mM HEPES, *p*H adjusted to 7.4). Microelectrodes were pulled in one stage from capillary glass (BBL with fiber; World Precision Instruments, Sarasota, FL) on a micropipette puller



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(model 700 C; David Kopf Instruments, Tujunga, CA). Electrode tips were broken to a diameter of approximately 10 $\mu$m, providing a resistance of 1–3 M$\Omega$, and filled with 3 M KCl. The cell was voltage clamped using a two-microelectrode oocyte voltage-clamp amplifier (OC725A; Warner Corp., New Haven, CT), connected to an IBM-compatible computer for data acquisition (DAS-8A/D conversion board: Keithley-Metrabyte, Traunton, MA) and analysis (OoClamp software[8]). All measurements were performed at a holding potential of −70 mV. Only cells exhibiting stable holding currents $<1$ $\mu$A during a 1-min equilibration period were included in the analysis. Membrane current was sampled at 125 Hz and recorded for 5 s before and 55 s after administration of the agonist, which allowed sufficient time for currents to return to baseline levels. Agonist (the $TXA_2$ mimetic U-46619) was delivered as a 30-$\mu$l aliquot during a period of 1–2 s using a hand-held micropipettor positioned approximately 3 mm from the oocyte. Agonist binding to $TXA_2$ receptors activates one or more heterotrimeric G proteins, which in turn modulate the enzyme-regulated synthesis of second messengers (fig. 1A). Specifically, the $G_q$ protein activates phospholipase C-$\beta$ (PLC-$\beta$), which cleaves membrane phosphatidylinositolbisphosphate to inositol-1-4-5-trisphosphate ($IP_3$) and diacylglycerol. The $IP_3$ activates receptor-channel complexes on intracellular $Ca^{2+}$ stores, resulting in an increase in intracellular $Ca^{2+}$ concentration. In the *Xenopus* oocyte, intracellular $Ca^{2+}$ opens endogenous membrane $Cl^-$ channels, resulting in a $Cl^-$ flux ($I_{Cl(Ca)}$) measured conveniently using the voltage-clamp technique (fig. 1). Responses were quantified by integrating the current trace (fig. 1B) and are reported in microcoulombs ($\mu$C). All experiments were performed at room temperature.

*Activation of Intracellular Signaling Pathway by Intracellular Microinjection of $IP_3$ and GTP$\gamma$S*

To study $IP_3$- and guanosine 5′-0-(2thiodiphosphate) (GTP$\gamma$S)-induced $I_{Cl(Ca)}$, a third micropipette was inserted into the voltage-clamped oocyte. Tips of intracellular micropipettes were beveled with a microgrinder (Narishige EG-6 Glass Electrode microgrinder; Narishige Instrument Laboratories, Tokyo, Japan). The pipette was connected to an automated microinjector (Nanoject, Drummond Scientific). Under voltage clamp, 30 nl of 2 mM $IP_3$ or 100 mM GTP$\gamma$S was injected, thereby activating the signaling pathway at the $IP_3$ receptor or the G protein, respectively (fig. 1A). Because the volume of an average *Xenopus* oocyte is approximately 500 nl, the injected volume approximated 5% of the oocyte volume. Therefore the estimated final concentrations were $IP_3$ 100 $\mu$M or GTP$\gamma$S 5 mM. These concentrations were chosen to result in $I_{Cl(Ca)}$ similar in size to those induced by $TXA_2$ agonist at its $EC_{50}$. Induced $I_{Cl(Ca)}$ were recorded 5 s before and 55 s after intracellular injection and analyzed as described before.

*Intracellular Heparin Injection*

To confirm that $I_{Cl(Ca)}$ induced by $TXA_2$ indeed resulted from $IP_3$ signaling, we injected the $IP_3$ receptor antagonist heparin (MW, 3,000 g/mol). An automated microinjector was used (Nanoject, Drummond Scientific). Oocytes were injected with 50 nl heparin (2 ng/nl; approximate final concentration, 0.2 ng/nl) at least 30 min before agonist application.

*Anesthetic Administration*

To determine the effects of halothane, isoflurane, and sevoflurane on $I_{Cl(Ca)}$ induced by U-46619 or intracellular mediators, anesthetic was bubbled through a reservoir filled with 40 ml Tyrode's solution for at least 10 min. Air at a flow rate of 500 ml/min was used as the carrier gas. After equilibration, the solution was perfused through the recording chamber, superfusing the oocyte at a flow rate of approximately 3 ml/min; measurements were obtained after 10 bath volumes had been exchanged. Anesthetic concentrations in the recording chamber were quantified by gas chromatography (Aerograph 940; Varian Analytical instruments, Walnut Creek, CA). Results were converted to concentration in liquid using partition coefficients in Tyrode's solution at 22°C (halothane $\lambda = 1.31$, isoflurane $\lambda = 1.08$, sevoflurane $\lambda = 0.39$), and to corresponding partial pressure (vol%) at room temperature.[9,10] Aqueous concentrations equivalent to 1 minimum alveolar concentration anesthetic in air were 0.43 mM for halothane, 0.44 mM for isoflurane, and 0.44 mM for sevoflurane. Although the halothane minimum alveolar concentration–equivalent concentration is approximately twice as high as that published by Franks and Lieb,[11] our values are similar to those reported by other investigators.[2,3,6,12] Each oocyte was exposed to a single concentration of anesthetic only.



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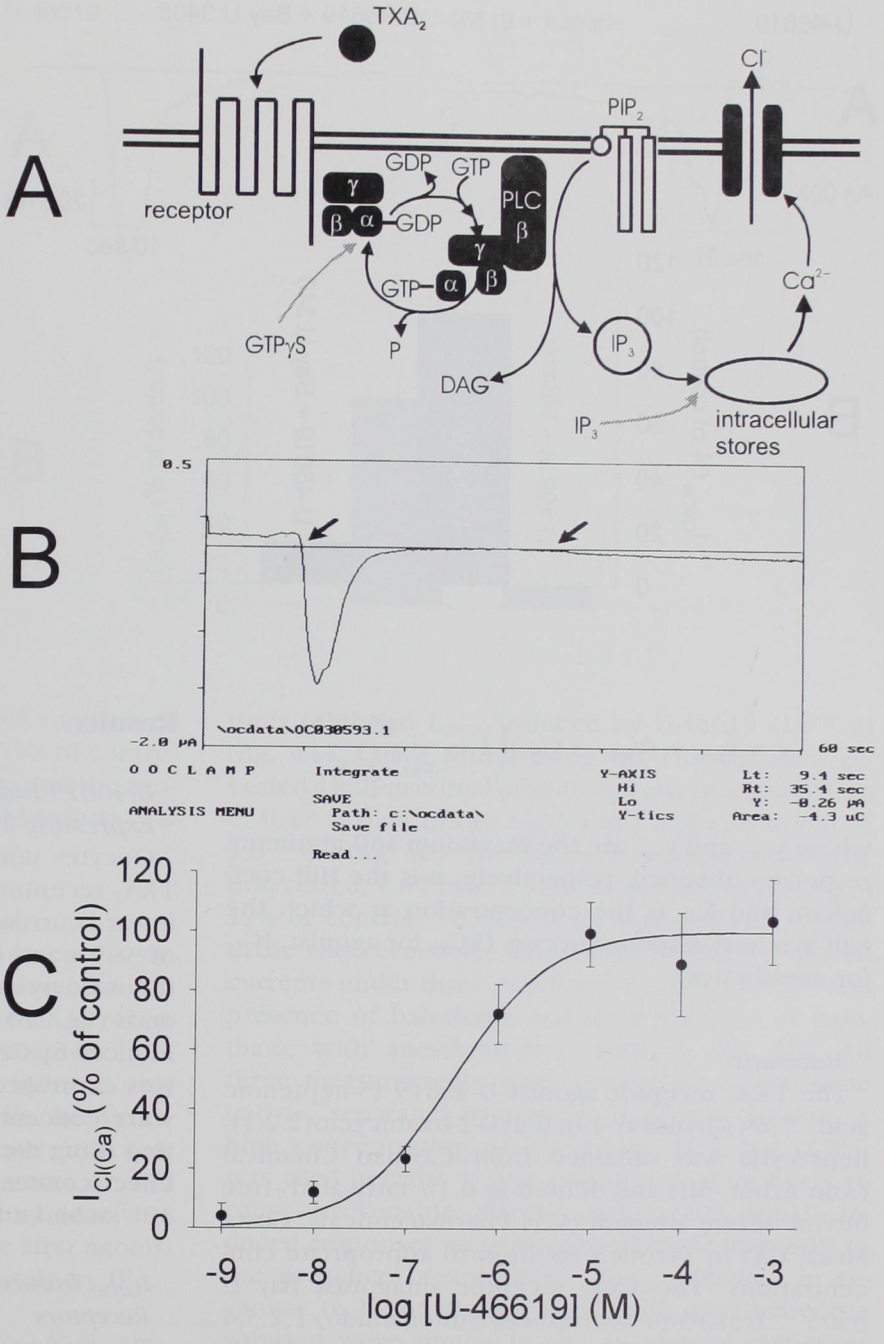


**Fig. 1.** ***Thromboxane $A_2$ ($TXA_2$) signaling in*** **Xenopus** ***oocytes.*** **(*A*) The signaling pathway linking $TXA_2$ receptor activation to chloride ($Cl^-$) channel opening. The double line represents the cell membrane. Binding of the ligand to the receptor activates G proteins (exchanging GDP for GTP). $G_q$ proteins activate phospholipase C (PLC-$\beta$), which cleaves membrane phosphatidylinositolbisphosphate to inositol 1-4-5 trisphosphate ($IP_3$) and diacylglycerol. $IP_3$ releases $Ca^{2+}$ from intracellular stores, increasing intracellular $Ca^{2+}$ concentration. Intracellular $Ca^{2+}$ opens endogenous membrane $Cl^-$ channels, resulting in a $Cl^-$ flux ($I_{Cl(Ca)}$). Shaded arrows indicate the site of action of intermediates microinjected to activate intracellularly the signaling pathway. (*B*) Example of $I_{Cl(Ca)}$ induced by U-46619 ($10^{-6}$ M) in oocyte-expressing $TXA_2$ receptors (screen capture using OoClamp[8]). The tracing shows a peak current of approximately 1.5 $\mu$A. The method of integration is indicated: A horizontal cursor was placed at baseline and the segment between 9.4 and 35.4 s was highlighted (here indicated by arrows) and integrated. Charge movement is 4.3 $\mu$C. (*C*) U-46619 activates $TXA_2$ receptors, recombinantly expressed in *Xenopus* oocytes, in a concentration-dependent manner. $I_{Cl(Ca)}$ induced by $10^{-3}$ M U-46619 ($y_{max}$, n = 28) was 13.4 ± 1.86 $\mu$C. Curve fitting using the Hill equation revealed a half maximal effect concentration of $3.2 \times 10^{-7} \pm 1.1 \times 10^{-7}$ M and a Hill coefficient of 0.8 ± 0.2 (n for each data point >7).**

*Data Analysis*

Results are reported as means ± SEM. Differences among treatment groups were analyzed using the Student's *t* test or the Mann-Whitney U test. If multiple comparisons were made, data were analyzed using analysis of variance followed by a *t* test corrected for multiple comparisons (Bonferroni). $P < 0.05$ was considered significant. Concentration-response curves were fit to the following logistic function, derived from the Hill equation:



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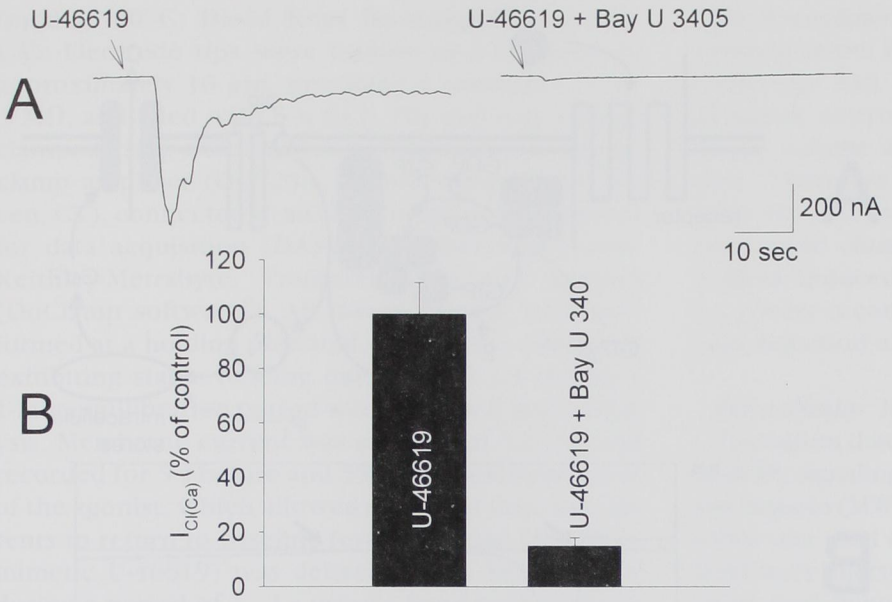


**Fig. 2.** ***Responses to U-46619 in thromboxane $A_2$ ($TXA_2$) receptor-expressing oocytes are inhibited by $TXA_2$ receptor antagonist Bay U 3405.*** **(*A*) Examples of responses induced by U-46619 in oocytes expressing $TXA_2$ receptors, in the absence and presence of the $TXA_2$ antagonist Bay U 3405 ($10^{-5}$ M). The charge movement in response to U-46619 ($10^{-6}$ M) is 6.4 $\mu$C in the absence, and 0.0 $\mu$C in the presence of $TXA_2$ receptor antagonist. (*B*) Treatment with $TXA_2$ antagonist Bay U 3405 ($10^{-5}$ M) suppresses the responses to U-46619 ($10^{-6}$ M) to 15% of control (n = 5 in each group).**

$$y = y_{min} + (y_{max} - y_{min})\left(1 - \frac{x^n}{x_{50}{}^n + x^n}\right)$$

where $y_{max}$ and $y_{min}$ are the maximum and minimum responses obtained, respectively; n is the Hill coefficient; and $X_{50}$ is the concentration at which the half-maximal response occurs ($EC_{50}$ for agonist, $IC_{50}$ for anesthetics).

*Materials*

The $TXA_2$ receptor agonist U-46619 (5-heptenoic acid, 7[6-(3-hydroxy-1-octenyl)-2-oxabicyclo[2.2.1]-hept-5-yl]) was obtained from Cayman Chemical (Ann Arbor, MI) and diluted in 0.1% fatty acid-free bovine serum albumin (ICN Pharmaceuticals, Costa Mesa, CA) in Tyrode's solution to appropriate concentrations. The $TXA_2$ receptor antagonist Bay U 3405 ((3R)-3-(4-fluorophenylsulfonamido)-1,2,3,4 tetrahydro [4$\alpha$,4b-3H]-carbazolepropanoic acid) was a gift from Bayer AG (Wuppertal, Germany) and was dissolved in the same manner. Halothane was from Halocarbon Laboratories (River Edge, NJ), sevoflurane was from Abbott International (Abbott Park, IL), and isoflurane was from Ohmeda (Liberty Corner, NJ). All other chemicals were from Sigma Chemical Company (St. Louis, MO).

## Results

*U-46619 Induces Inward Currents in Oocytes Expressing $TXA_2$ Receptors*

Oocytes injected with 5 ng mRNA encoding the $TXA_2$ receptor responded to U-46619 with transient inward currents. The current developed after a delay of 3-5 s and consisted of a fast inward component followed by a fluctuating relaxation over several seconds (fig. 1B). These responses are typical for $I_{Cl(Ca)}$ induced by $Ca^{2+}$-signaling G protein-coupled receptors expressed in *Xenopus* oocytes.[3] The responses were concentration dependent (fig. 1C). Curve fitting using the Hill equation revealed a half-maximal effect concentration ($EC_{50}$) of $3.2 \times 10^{-7} \pm 1.1 \times 10^{-7}$ M and a Hill coefficient of $0.8 \pm 0.2$.

*$I_{Cl(Ca)}$ Induced by U-46619 Is Mediated by $TXA_2$ Receptors*

To confirm that the $I_{Cl(Ca)}$ induced by U-46619 is indeed mediated by recombinantly expressed $TXA_2$ receptors, we applied the agonist (1) to uninjected cells to determine if endogenous $TXA_2$ receptors were present and (2) to cells expressing the $TXA_2$ receptor, 1-2 min after application of the specific $TXA_2$ receptor antagonist Bay U 3405 ($10^{-5}$ M). U-46619 ($10^{-4}$ M) had no effect in uninjected cells (data



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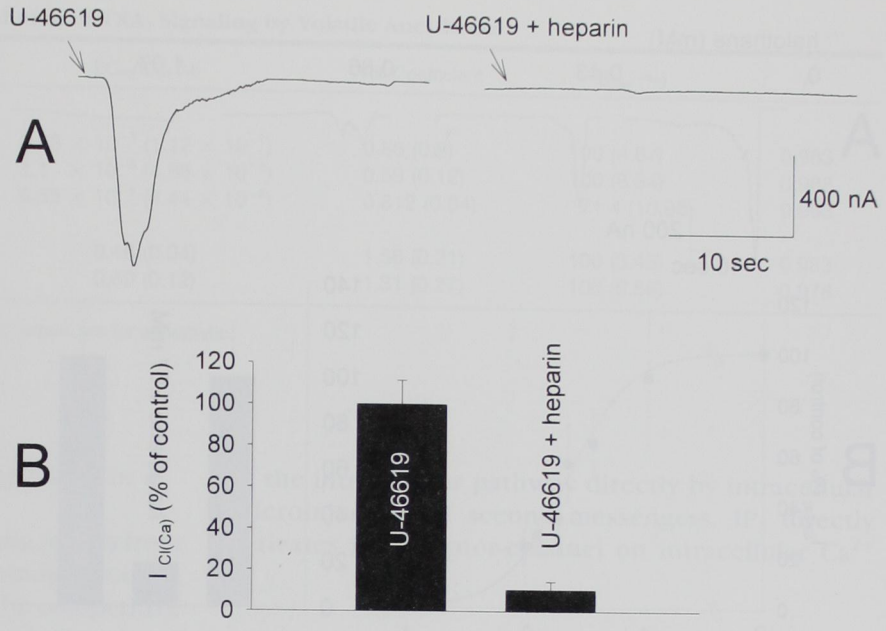


**Fig. 3.** ***Responses to U-46619 in thromboxane $A_2$ ($TXA_2$) receptor-expressing oocytes are mediated by inositol 1-4-5 trisphosphate ($IP_3$).*** **(*A*) Examples of responses induced by U-46619 ($10^{-6}$ M) in oocytes expressing $TXA_2$ receptors, in the absence and presence of intracellular $IP_3$ antagonist heparin (2 ng/nl, 50 nl). The charge movement in response to U-46619 is 5.8 $\mu$C in the absence and 0.5 $\mu$C in the presence of intracellular heparin. (*B*) Intracellular microinjection of the selective $IP_3$ antagonist heparin (2 ng/nl, 50 nl) inhibits U-46619 ($10^{-6}$ M)-induced $I_{Cl(Ca)}$ to 10% of control (n = 5 in each group).**

not shown). Treatment with Bay U 3405 suppressed the responses to U-46619 ($10^{-6}$ M) to 15% of control (fig. 2). Together these findings confirm that the agonist indeed signaled through recombinantly expressed $TXA_2$ receptors.

*$IP_3$ Mediates the $TXA_2$ Receptor Signal*

To determine if the expressed $TXA_2$ receptors signaled through $IP_3$ release (fig. 1A), we injected 100 ng heparin into oocytes expressing the $TXA_2$ receptor. Heparin selectively blocks $IP_3$ receptors.[13] U-46619 ($10^{-6}$ M), applied 30 min later, induced only minute currents in heparin-injected cells (fig. 3): Responses were suppressed to 10% of control. In contrast, control injections with sterile water or KCl (150 mM) did not affect $TXA_2$ signaling (data not shown). Thus intracellular $Ca^{2+}$ release after agonist application is mediated almost completely by $IP_3$.

*$TXA_2$ Signaling Is Inhibited Reversibly by Clinically Relevant Halothane Concentrations*

After confirming that the receptor was functional, that the agonist was acting selectively on expressed receptors, and that the intracellular signaling pathway was as described for this receptor (fig. 1A), we tested the ability of halothane to interfere with $TXA_2$ signaling. Halothane at clinically relevant concentrations inhibited $I_{Cl(Ca)}$ induced by U-46619 ($10^{-6}$ M) (fig. 4A). Curve fitting using the Hill equation revealed a half-maximal inhibitory concentration ($IC_{50}$) of 0.47 ± 0.05 mM (0.83%) and a Hill coefficient of 1.6 ± 0.2 (fig. 4B). The highest halothane concentration tested, 1.07 mM (2.5%), suppressed signaling to 17% of control. To determine if the effect of halothane was reversible, we studied U-46619–induced currents under three conditions: in the absence and presence of halothane and after washout of halothane with anesthetic-free solution (fig. 4C). All three measurements were performed in the same oocyte, separated by washes of at least 10 min. A high concentration of halothane (1.07 mM, 2.5%) was used because it was considered more likely to cause irreversible effects. Although U-46619–induced responses were depressed more than 80% in the presence of anesthetic, $I_{Cl(Ca)}$ obtained in response to U-46619 after wash with anesthetic-free solution were similar in size to control responses (fig. 4C), indicating that halothane's effect is reversible.

*$TXA_2$ Signaling Is Reversibly Inhibited by Clinically Relevant Isoflurane Concentrations*

Next we investigated isoflurane's effect on $TXA_2$ signaling. Isoflurane, at clinically relevant concentra-



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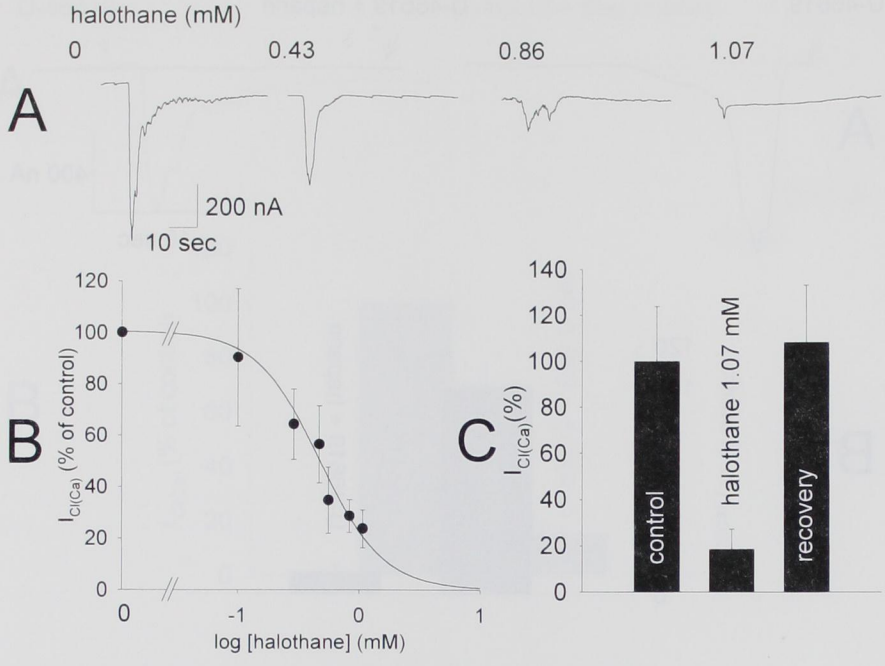


**Fig. 4.** ***Halothane reversibly inhibits thromboxane $A_2$ ($TXA_2$) receptor functioning.*** **(*A*) Examples of $I_{Cl(Ca)}$ induced by U-46619 ($10^{-6}$ M) in oocytes expressing $TXA_2$ receptors. The charge movement in response to U-46619 is 8.6 $\mu$C. Halothane concentrations are indicated and correspond to 1, 2, and 3 minimum alveolar concentrations, resulting in charge movements of 3.9, 1.7, and 0.8 $\mu$C, respectively. (*B*) Halothane inhibits $I_{Cl(Ca)}$ responses to $TXA_2$ agonist U-46619 ($10^{-6}$ M) in a concentration-dependent manner. Curve fitting using the Hill equation revealed a half-maximal inhibitory concentration ($IC_{50}$) of 0.47 ± 0.05 mM (0.83%) and a Hill coefficient of 1.6 ± 0.2. (*C*) $I_{Cl(Ca)}$ induced by U-46619 ($10^{-6}$ M) to determine the reversibility of halothane inhibition. Three consecutive measurement were made in each oocyte. The first bar represents control measurements (9.36 ± 1.86 $\mu$C). The second bar shows the inhibitory effect of 1.07 mM halothane (2.5%). $I_{Cl(Ca)}$ was reduced to 17% of control (1.59 ± 0.86 $\mu$C). The third bar shows recovery after 10 min of perfusion with Tyrode's solution without anesthetic (9.58 ± 1.98 $\mu$C).**

tions, inhibited U-46619 ($10^{-6}$ M) - induced $I_{Cl(Ca)}$ (fig. 5A). Curve fitting revealed an $IC_{50}$ of 0.69 ± 0.12 mM (1.7%, fig. 5B) and a Hill coefficient of 1.3 ± 0.3. The highest isoflurane concentration tested (1.32 mM, 3.3%) suppressed signaling to 29.5% of control. The inhibitory effect was reversible (fig. 5C).

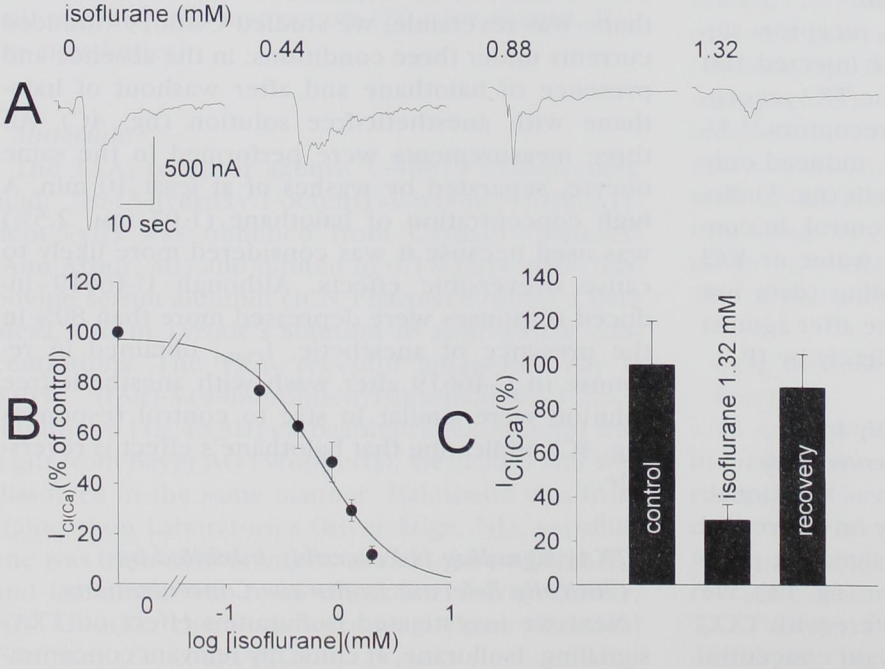


**Fig. 5.** ***Isoflurane reversibly inhibits thromboxane $A_2$ ($TXA_2$) receptor functioning.*** **(*A*) Examples of $I_{Cl(Ca)}$ induced by U-46619 ($10^{-6}$ M) in oocytes expressing $TXA_2$ receptors. The charge movement in response to U-46619 is 7.4 $\mu$C. Isoflurane concentrations are indicated and correspond to 1, 2, and 3 minimum alveolar concentrations, resulting in charge movements of 5.4, 3.6, and 1.8 $\mu$C, respectively. (*B*) Isoflurane inhibits U-46619 ($10^{-6}$ M)-induced $I_{Cl(Ca)}$ in a concentration-dependent manner. Curve fitting using the Hill equation revealed a half-maximal inhibitory concentration ($IC_{50}$) of 0.69 ± 0.12 mM (1.7%) and a Hill coefficient of 1.3 ± 0.3. (*C*) $I_{Cl(Ca)}$ induced by U-46619 ($10^{-6}$ M) to determine reversibility of isoflurane inhibition. Three consecutive measurements were made in each oocyte. The first bar represents control measurements (8.68 ± 1.73 $\mu$C). The second bar shows the inhibitory effect of 1.32 mM isoflurane (3.3%). $I_{Cl(Ca)}$ was reduced to 29.5% of control (2.56 ± 0.62 $\mu$C). The third bar shows recovery after 10 min of perfusion with Tyrode's solution without anesthetic (7.8 ± 1.32 $\mu$C).**



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**Table 1. Pharmacologic Parameters for Inhibition of $TXA_2$ Signaling by Volatile Anesthetics**

| | $EC_{50}/IC_{50}$ (M) | Hill Coefficient | $E_{max}$ (%) | $r^2$ |
|---|---|---|---|---|
| Concentration/response relationship | | | | |
| Control | $3.23 \times 10^{-7}$ ($1.12 \times 10^{-7}$) | 0.86 (0.2) | 100 (4.87) | 0.983 |
| Halothane (0.56 mM) | $1.1 \times 10^{-5}$ ($4.86 \times 10^{-6}$) | 0.59 (0.12) | 100 (8.94) | 0.988 |
| Isoflurane (0.7 mM) | $5.33 \times 10^{-7}$ ($4.44 \times 10^{-8}$) | 0.812 (0.04) | 71.4 (10.98) | 0.993 |
| Concentration/inhibition relationship | | | | |
| Halothane | 0.46 (0.04) | 1.56 (0.21) | 100 (3.45) | 0.983 |
| Isoflurane | 0.69 (0.12) | 1.31 (0.27) | 100 (6.58) | 0.978 |

Data are mean (SEM). $EC_{50}$ values are for U-46619, $IC_{50}$ values are for anesthetic.

*Halothane Acts in a Competitive, Isoflurane in a Noncompetitive Manner*

If anesthetic inhibition of $TXA_2$ signaling results from hydrophobic interactions at the ligand-binding pocket, we would expect that the effect would be competitive (that is, fully reversible by high agonist concentrations) in nature. In contrast, a site of action elsewhere in the signaling pathway would likely be noncompetitive (that is, not fully reversible by high agonist concentrations). To determine if halothane and isoflurane act as competitive or noncompetitive antagonists on $TXA_2$ signaling, the concentration-response relation for U-46619 was determined in the presence of halothane 0.56 mM (1%, close to halothane's $IC_{50}$) or isoflurane 0.7 mM (1.75%, close to isoflurane's $IC_{50}$). Halothane acted in a competitive manner: it did not affect $E_{max}$ but caused a parallel shift of the concentration-response relation to the right. This increased the U-46619 $EC_{50}$ by approximately two orders of magnitude to $1.1 \times 10^{-5}$ M (table 1). In contrast, isoflurane acted in a noncompetitive manner: It did not affect the U-46619 $EC_{50}$ ($5.3 \times 10^{-7}$ M) but decreased $E_{max}$ to 70% of control (table 1, fig. 6).

*Sevoflurane Is Without Effect on $TXA_2$ Signaling*

We also tested the ability of sevoflurane to inhibit $TXA_2$ signaling. In contrast to the other anesthetics tested, sevoflurane, at concentrations up to 5% (0-1.3 mM, 2.92 minimum alveolar concentration),[14] did not affect $TXA_2$ signaling (fig. 7).

*Halothane, Isoflurane, and Sevoflurane Have No Effect on Intracellular Signaling Pathways*

To determine the site of action of halothane on the $TXA_2$ signaling pathway, we activated segments of the intracellular pathway directly by intracellular microinjection of second messengers. $IP_3$ directly activates its receptor-channel on intracellular $Ca^{2+}$

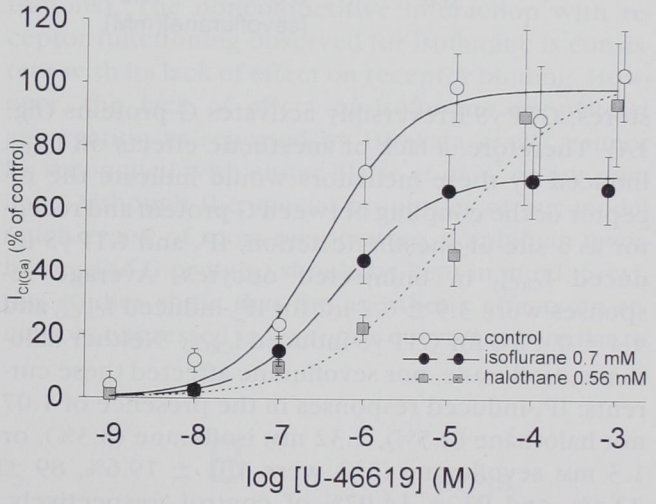


**Fig. 6. *Halothane inhibits thromboxane $A_2$ ($TXA_2$) signaling in a competitive manner, whereas isoflurane inhibits in a noncompetitive manner.*** **The inhibitory effects of halothane and isoflurane on $TXA_2$ signaling are different. Halothane (0.56 mM; 1%) shifts the agonist concentration-response curve for U-46619 to the right. Maximal $I_{Cl(Ca)}$ at $10^{-3}$ M U-46619 was $12.7 \pm 1.73$ $\mu$C (n = 10). Curve fitting using the Hill equation revealed an increase of the U-46619 median effective concentration ($EC_{50}$) by two orders of magnitude: from $3.2 \pm 10^{-7} \pm 1.1 \times 10^{-7}$ M to $1.0 \times 10^{-5} \pm 0.3 \pm 10^{-5}$ M. The Hill coefficient and $E_{max}$ were unchanged, suggesting that halothane acts as a competitive antagonist. (The control concentration–response curve is from figure 1C). In contrast, isoflurane did not change the Hill coefficient ($0.82 \pm 0.04$) or $EC_{50}$ ($5.33 \pm 10^{-7} \pm 4.4 \times 10^{-8}$ M) but did significantly decrease $E_{max}$ (70% of same day control), suggesting that isoflurane acts as a noncompetitive antagonist.**



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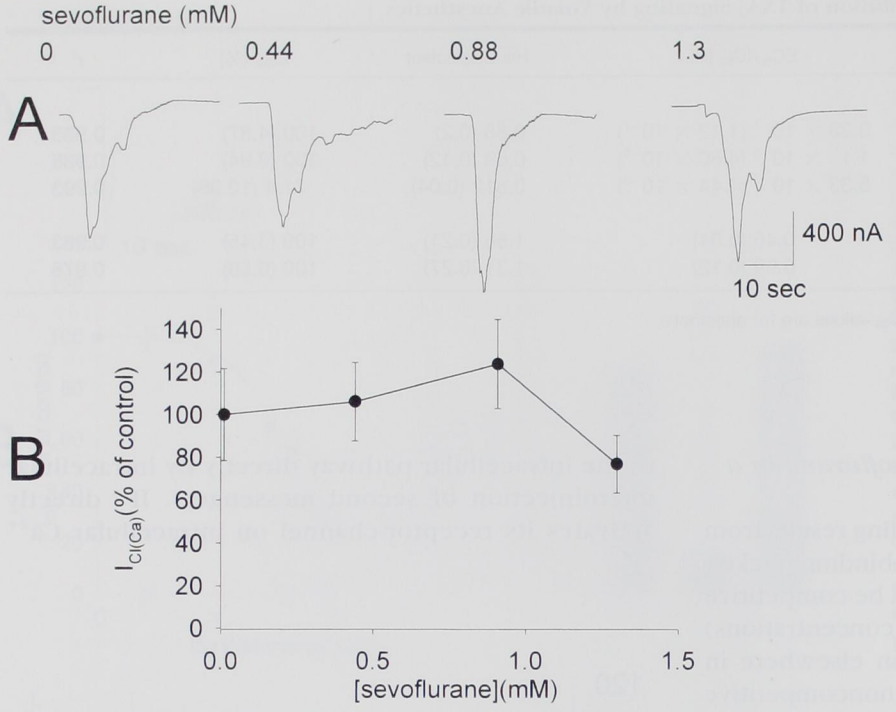


**Fig. 7.** ***Clinically relevant concentrations of sevoflurane have no effect on thromboxane $A_2$ ($TXA_2$) receptor functioning.*** **(*A*) Examples of $I_{Cl(Ca)}$ induced by U-46619 ($10^{-6}$ M) in oocytes expressing $TXA_2$ receptors. The charge movement in response to U-46619 is 8.3 $\mu$C. Sevoflurane concentrations are indicated and correspond to 1, 2, and 3 minimum alveolar concentration, resulting in charge movements of 9.6, 8.7, and 9.1 $\mu$C, respectively. (*B*) Sevoflurane at clinically relevant concentrations has no inhibitory effect on $I_{Cl(Ca)}$ induced by $TXA_2$ agonist U-46619 ($10^{-6}$ M).**

stores; GTP$\gamma$S irreversibly activates G proteins (fig. 1A). Therefore, a lack of anesthetic effects on $I_{Cl(Ca)}$ induced by these mediators would indicate the receptor or the coupling between G protein and receptor as a site of anesthetic action. $IP_3$ and GTP$\gamma$S induced $I_{Cl(Ca)}$ in uninjected oocytes. Average responses were 3.9 ± 0.2 $\mu$C for $IP_3$-induced $I_{Cl(Ca)}$ and 4.4 ± 0.4 $\mu$C for GTP$\gamma$S-induced $I_{Cl(Ca)}$. Neither halothane, isoflurane, nor sevoflurane affected these currents: $IP_3$-induced responses in the presence of 1.07 mM halothane (2.5%), 1.32 mM isoflurane (3.3%), or 1.3 mM sevoflurane (5%) were 101 ± 19.6%, 89 ± 22.4%, and 93 ± 14.07% of control, respectively. GTP$\gamma$S-induced responses were 131 ± 25.4% of control in the presence of halothane, 122 ± 18.6% in the presence of isoflurane, and 120 ± 32.5% of control in the presence of sevoflurane (not significantly different from control; fig. 8). When 10-fold greater or 10-fold smaller concentrations of $IP_3$ and GTP$\gamma$S were injected, the anesthetics were similarly without effect (data not shown).

## Discussion

Our findings show that clinically relevant concentrations of halothane and isoflurane inhibit functioning of recombinantly expressed $TXA_2$ receptors. Halothane acts in a competitive, and isoflurane in a noncompetitive manner. In contrast, sevoflurane does not inhibit $TXA_2$ signaling. Neither anesthetic affects intracellular signaling pathways.

### *Thromboxane $A_2$ Signaling*

Thromboxane $A_2$ is a potent stimulator of platelet aggregation[5,6,15] and a constrictor of vascular[5,16] and respiratory smooth muscle.[17] Its function is counterbalanced with that of prostacyclin, which inhibits platelet aggregation and elicits vasorelaxation. Disruption of this balance in favor of $TXA_2$ has been suggested to play a role in thrombosis, asthma, and unstable angina, as well as in myocardial infarction.[18] Thus $TXA_2$ receptor antagonists are of considerable therapeutic importance.[17] Therefore acute inhibitory effects of anesthetics on $TXA_2$ signaling may be beneficial; in addition, short-term modulation of $TXA_2$ signaling during anesthesia may result in long-term beneficial effects as well.[19]

Hirakata *et al.*[20] determined the amino acid sequence of the human $TXA_2$ receptor by cloning it from a human placental cDNA library. Nüsing *et al.*[12] recently cloned the gene for the human $TXA_2$ recep-

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tor and found no evidence for additional genes. Thus only one $TXA_2$ receptor type appears to exist. This is unusual, because for most other members of the G protein - coupled receptor superfamily the existence of several receptor subtypes has been demonstrated. The present study had the advantage that our findings are not subtype dependent.

*Anesthetic Effects on $TXA_2$ Signaling*

Data on the interactions between volatile anesthetics and $TXA_2$ signaling are contradictory. Hirakata *et al.*[6] demonstrated interference of halothane and sevoflurane with the $TXA_2$ synthetic pathway in human platelets. As as result, halothane (0.49 - 1.25 mM) and sevoflurane (0.13 - 1.3 mM) blocked secondary aggregation induced by epinephrine or adenosine diphosphate. Isoflurane (0.28 - 0.84 mM) had no significant effect. Whereas sevoflurane (3 mM) and isoflurane (2.5 mM) had minimal effects on platelet binding of the $TXA_2$-receptor antagonist [$^3$H]S145 (5Z-7-(3-endo([ring-4-$^3$H]phenyl)sulphonylamino-[2.2.1.]bicyclohept-2-exo-yl)heptenoic acid), halothane (3.3 mM) suppressed binding. It should be noted that the halothane concentration required to interfere with binding was much greater than that required to block aggregation. Scatchard analysis of [$^3$H]S145 binding showed that sevoflurane affected neither $B_{max}$ nor $K_d$, whereas halothane (3.3 mM) markedly increased $K_d$ without significantly altering $B_{max}$. The authors concluded that (1) halothane interfered both with $TXA_2$ - receptor interaction and with cyclo-oxygenase function, (2) sevoflurane inhibited only cyclo-oxygenase and (3) isoflurane seemed to affect neither cyclo-oxygenase nor $TXA_2$ receptor binding. In contrast, Blaise *et al.*,[5] while observing similar effects of halothane, reported different results for isoflurane: In their model, isoflurane inhibited platelet aggregation induced by adenosine diphosphate, collagen, epinephrine, and arachidonic acid. These findings were confirmed in a second model: vascular smooth muscle contraction induced by U-46619 and potassium chloride.

Because of these inconsistencies, and because functioning of the $TXA_2$ receptor *per se* had not been studied, we did this investigation. We found no interference of sevoflurane with $TXA_2$ signaling, in agreement with the lack of receptor binding observed by Hirakata *et al.*[6] Halothane did inhibit receptor functioning in a competitive manner, consistent with its effects on receptor binding (although the functional effects are notable at much lower concentrations). The noncompetitive interaction with receptor functioning observed for isoflurane is consistent with its lack of effect on receptor binding. However, the lack of effect of isoflurane on platelet aggregation as reported by Hirakata *et al.*[6] cannot be reconciled with either Blaise *et al.*'s[5] or our own data. Although the special properties of our model (such as use of room temperature, amphibian membrane, and G protein) should be kept in mind, previous studies show that the anesthetic effects on receptors expressed in *Xenopus* oocytes are similar to

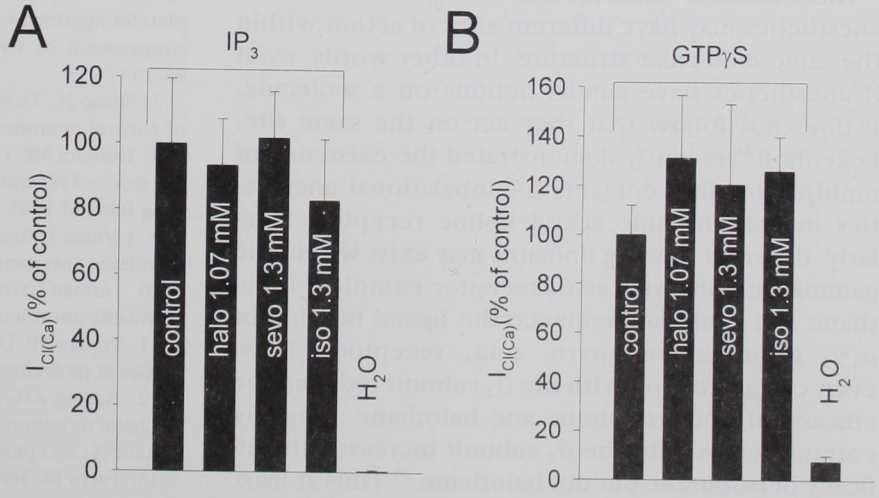


**Fig. 8.** ***The intracellular pathway is not modulated by volatile anesthetics.*** **(*A*) $I_{Cl(Ca)}$ induced by intracellular microinjection of GTPγS are not inhibited by sevoflurane, isoflurane, or halothane at concentrations of 1.3 mM, 1.3 mM, and 1.07 mM, respectively. Intracellular injection of water does not induce $I_{Cl(Ca)}$. (*B*) Intracellular microinjection of the second messenger inositol 1-4-5 trisphosphate ($IP_3$) induces $I_{Cl(Ca)}$. Currents are not inhibited by halothane (1.07 mM), isoflurane (1.32 mM), or sevoflurane (1.3 mM).**



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those observed in other models.[2,3,21,22] In addition, our data are consistent with those of Blaise *et al.*[5]

*Site of Action*

Our experiments with microinjected intermediates show that the intracellular signaling pathway is unaffected by the anesthetics. Therefore, the receptor itself is the most likely site of action. Because halothane's effect is competitive and it interferes with ligand binding,[6] its action is probably at the ligand-binding site. Because halothane is the most lipid soluble of the three anesthetics tested (oil-water partition coefficient 310[23]), we hypothesize that the site of halothane's action is hydrophobic. Yamamoto *et al.*[15] modeled the structure of the $TXA_2$ receptor and identified several amino acid residues likely involved in ligand binding, as well as a large hydrophobic pocket among these amino acids. Based on this combination of data, we postulate that halothane interacts with this hydrophobic pocket.

In contrast, inhibition by isoflurane is noncompetitive, and the anesthetic does not interfere with ligand binding.[6] Therefore its (allosteric) site of action is unlikely to be at the ligand-binding pocket. Its lipophilicity (oil-water partition coefficient 170[23]) is less than that of halothane, making it a less suitable candidate for interaction with the lipophilic pocket. In agreement, sevoflurane (oil-water partition coefficient 32[23]) was completely without effect. The exact site of action of isoflurane cannot be determined from the present studies. However, mutation analysis might make localization of this site possible.

These findings reinforce the concept that different anesthetics may have different sites of action within the same molecular structure. In other words, even if anesthetics have similar actions on a molecule, it does not follow that they act on the same site. Eckenhoff[24] recently demonstrated the existence of multiple binding domains for inhalational anesthetics in the nicotinic acetylcholine receptor. Similarly, different binding domains may exist within the gamma-aminobutyric $acid_A$ receptor complex. Halothane and isoflurane enhance the ligand binding of $\alpha_1\gamma_2$ gamma-aminobutyric $acid_A$ receptors. However, cotransfection with the $\beta_2$ subunit reduced the efficacy of both isoflurane and halothane, whereas cotransfection with the $\beta_3$ subunit increased the efficacy of isoflurane but not halothane.[25] Thus at least several signaling molecules may have multiple, separate sites of action for volatile anesthetics.

## Conclusions

Whereas halothane and isoflurane inhibit $TXA_2$ receptor functioning, halothane acts in a competitive and isoflurane acts in a noncompetitive manner. In contrast, sevoflurane has no effect. The site of action appears to be the receptor molecule itself. The site of halothane's action is most likely the hydrophobic pocket in the ligand-binding domain. In contrast, isoflurane most likely acts at an allosteric site.

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