Differential Effect of Halothane and Forskolin on Platelet Cytosolic Ca^{2+} Mobilization and Aggregation

François Corbin, M.Sc.,* Gilbert Blaise, M.D.,† Rémy Sauvé, Ph.D.‡

Background: Previous works have suggested that the impairment of platelet aggregation by halothane was partly related to a stimulation of cyclic adenosine monophosphate (cAMP) production, to an inhibitory effect on Ca^{2+} signaling, or both. Intracellular Ca^{2+} measurements therefore were undertaken, first to determine the critical steps in the platelet Ca^{2+} signaling cascade most likely to be affected by halothane or by an increase in cAMP production, and second to establish if the effect of halothane involves aggregation-related biochemical pathways triggered by an increase in internal Ca^{2+}.

Methods: Human washed platelets were treated with halothane or forskolin for 5 min before application of either platelet-activating factor, thrombin, U46619, or thapsigargin. The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured with the fluorescent Ca^{2+} indicator fura-2. Nephelometric measurements were also performed to assay the aggregation process.

Results: Our results indicate that pretreating platelets with halothane leads to a partial impairment of the $[Ca^{2+}]_i$ increase induced either by U46619, thrombin, or platelet-activating factor, but this had no significant effect on the $[Ca^{2+}]_i$ response triggered by thapsigargin. In addition, our results show that halothane inhibits platelet aggregation triggered by U46619, but not by thapsigargin. Conversely, forskolin completely inhibited the $[Ca^{2+}]_i$ response to U46619 and thapsigargin and prevented platelet aggregation induced by both agonists.

Conclusions: These results suggest that halothane and cAMP exert their effects on platelet aggregation and Ca^{2+} signaling through different mechanisms, and that halothane cannot impair platelet aggregation independently of phospholipase C stimulation. (Key words: Bleeding; phospholipase C; second messenger; thrombus; time.)

* Graduate Student, Department of Physiology.

† Professor, Department of Anesthesia.

‡ Professor, Department of Physiology.

Address reprint requests to Dr. Sauvé: Département de Physiologie, Université de Montréal, C.P. 6128, Succ. A, Montréal (Québec), Canada, H3C 3J7. Address electronic mail to: sauvere@alize.ere.umontreal.ca

AMONG volatile anesthetics, halothane is unique in its ability to alter platelet function at clinically relevant concentrations.[1] In fact, halothane increases bleeding time *in vivo*[2] and impairs platelet aggregation *in vitro*.[3,4] More recently, it was reported that halothane protects against thrombus formation in stenosed coronary arteries in the dog.[5] However, the cellular mechanism underlying the action of halothane on platelet function still remains poorly circumscribed.

Platelet aggregation is a complex phenomenon that likely involves several intracellular biochemical pathways.[6] There is increasing evidence that internal Ca^{2+} stores play a central role in the response of platelets to activating agents.[7] It is thus possible that part of the observed effect of halothane on platelet aggregation results from an impaired Ca^{2+} response. In fact, halothane is already known to affect Ca^{2+} signaling in many cell types,[8-10] including human platelets.[11] There are, however, several possible ways by which halothane can interfere with Ca^{2+} signaling and platelet aggregation. For instance, halothane could affect the production of second messengers linked to Ca^{2+} mobilization, or inhibit directly the internal Ca^{2+} release plus agonist-induced Ca^{2+} influx initiated after platelet stimulation. In the latter case, the halothane effect would not require phospholipase C activation. In addition, halothane might affect the biochemical pathways that are triggered by internal Ca^{2+} and that lead to platelet aggregation. Under these conditions, halothane would impair platelet aggregation independently of the mechanisms responsible for the increase in cytosolic Ca^{2+}.

Several studies have also provided evidence that cyclic AMP (cAMP) is involved in the action of halothane on many cell types, including platelets.[12,13] In these preparations, halothane was shown to promote cAMP formation by stimulating adenylate cyclase without af-

fecting phosphodiesterases.[11] It is known that cAMP is one of the most potent platelet function inhibitors, and investigators have proposed that the inhibitory action of halothane on platelet aggregation resulted from an increased level of cAMP.[13] Because cAMP impairs Ca^{2+} mobilization in platelets,[14] an effect of halothane on aggregation may also arise from an enhanced cAMP production, which would in turn affect internal Ca^{2+} mobilization.

Thus the purpose of this study was to determine more precisely the mechanism by which halothane affects Ca^{2+} signaling and platelet aggregation. Our results indicate first that halothane and cAMP exert their inhibitory effect through distinct mechanisms, and second that the action of halothane in these cells requires phospholipase C stimulation.

Materials and Methods

Solutions and Drugs

The acid-citrate dextrose anticoagulant was composed of 85 mM sodium citrate, 78 mM citric acid, and 111 mM dextrose. The control solution was composed of 10 mM HEPES (*p*H 7.4), 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 10 mM dextrose. Fura-2-AM, BAPTA (1,2-bis(2-aminophenoxy)ethane N,N,N′,N′-tetraacetic acid), acetylsalicylic acid, and digitonin were purchased from Sigma Chemical Company (St. Louis, MO); U46619 was from Pharmacia and Upjohn (Kalamazoo, MI); thrombin (human plasma), forskolin, and platelet activating factor C-16 (1-O-palmityl-2-acetyl-sn-glycero-3-phosphocholine) were obtained from Calbiochem (San Diego, CA). Stock solutions of 24 mM halothane (Ayerst, Montréal, Québec, Canada) were prepared by vigorous stirring of halothane dissolved in control solutions for 2 h.

Platelet Preparation

Fura-2–loaded platelets were prepared according to the method of Sage *et al.*,[15] with minor modifications. Blood from healthy nonmedicated volunteers was drawn in plastic tubes containing 15% vol/vol acid-citrate dextrose anticoagulant. Blood was centrifuged at 350g for 10 min, and the resulting platelet-rich plasma was treated with 100 μM aspirin to ensure that the observed agonist-evoked effects did not result from the secondary formation of prostaglandins and thromboxanes. No phosphodiesterase inhibitors were used. Platelets in platelet-rich plasma were loaded with the fluo-

rescent probe fura-2-AM (4 μM) for 40 min at room temperature. They were sedimented by centrifugation at 437.5g for 15 min and resuspended in the control solution to achieve an approximate concentration of 1.2×10^6 platelets per milliliter.

Experimental Procedure

Washed platelets (1.4 ml) were introduced into a 1.8-ml gas-tight silicone-coated glass bottle that serves as a "gas-tight spectrofluorimeter cuvet." $CaCl_2$ was added to a final concentration of 1 mM 5 min before fura-2 measurements. After the addition of Ca^{2+}, 100 μl of the 24 mM halothane stock solution were injected into the cuvet using a gas-tight syringe, for a final concentration of 1.6 mM. This concentration of halothane corresponds to a gaseous partial pressure of 5% at 37°C, but to 3% in the current case because the experiments were done at 22°C.[16] The effective halothane concentration was tested in experiments in which the halothane content of control solutions was measured by gas chromatography, as previously described.[17] A similar procedure was used in experiments in which platelets were pretreated with forskolin (0.1 to 10 μM). Platelets were then stimulated with either 1 μM U46619, 10 nM platelet-activating factor, 1 U/ml thrombin, or 1 μM thapsigargin. In some experiments, 10 mM of the Ca^{2+} chelating agent BAPTA was added 50 s before the addition of the platelet stimulator to elicit a response in the absence of external Ca^{2+}.

Fluorometric Measurements

Fluorometric measurements were performed using a dual-excitation spectrofluorometer (Spex Fluorolog II, Spex Industries, Edison, NJ). The excitation wavelengths were set at 340 nm and 380 nm, and emission was monitored at 505 nm. The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated from the ratio of the fluorescence measured at 340 nm and 380 nm.[18] The ratios, R_{min} and R_{max}, were obtained by measuring the fluorescence in 5 mM EGTA plus digitonin (50 μM) at *p*H 9.0 in the absence and the presence of 10 mM $CaCl_2$, respectively.

Qualitative Aggregation Measurements

For the aggregation measurements, washed platelets were prepared as described before and the same protocols were applied. Nephelometric measurements were used to assay the aggregation, with the increase in light transmission proportional to the extent of the aggregation. All experiments were done at room temperature.
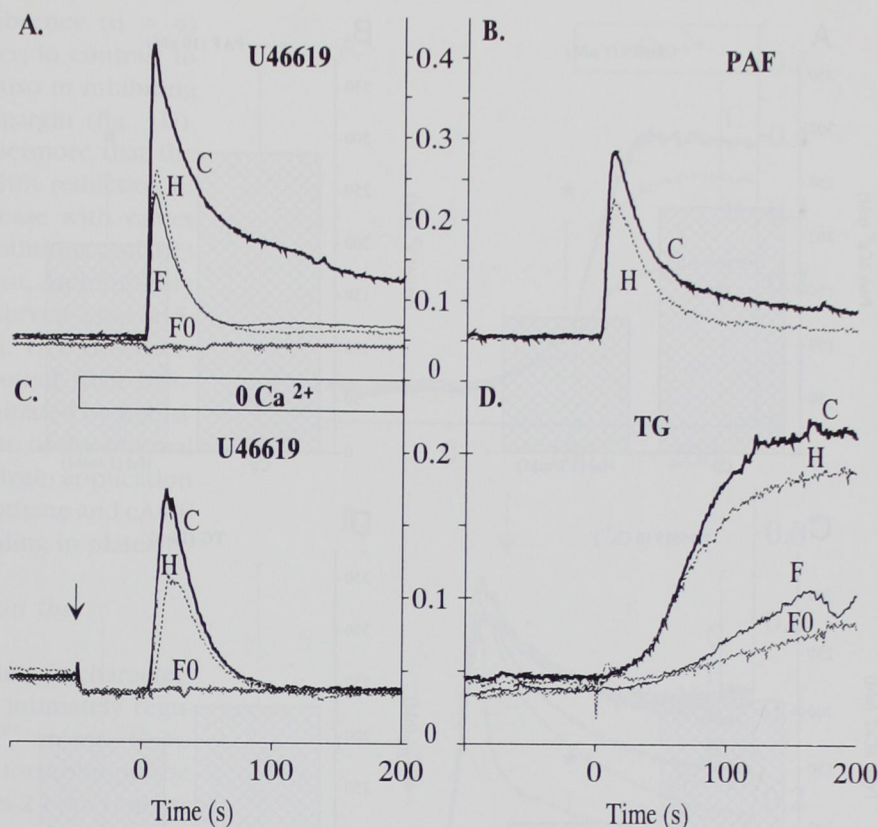
Fig. 1. Examples of $[Ca^{2+}]_i$ measurements on fura-2–loaded platelets. (*A*) Platelets were bathed for 5 min in solutions containing either 1.6 mM halothane (H) or 0.1 μM (F) forskolin to 1 μM (F0) forskolin before the addition of 1 μM U46619. (*B*) Effect of a 5-min pretreatment with 1.6 mM halothane (H) on the Ca^{2+} response in platelets stimulated by 10 nM platelet-activating factor (PAF). (*C*) Platelet Ca^{2+} response in zero-external Ca^{2+} conditions. Platelets were treated for 5 min with either 1.6 mM halothane (H) or 1 μM forskolin (F0) before the addition of 1 μM U46619. The Ca^{2+} chelator BAPTA (2 mM) was added 50 s before U46619 (*arrow*). (*D*) Differential effect of 0.1 μM (F) forskolin to 1 μM forskolin (F0) and halothane (H) pretreatment (5 min) on cells stimulated with the sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin (TG; 1 μM). The experiments were done in the presence of 1 mM external Ca^{2+}. Control curves are labeled as C. The cytosolic Ca^{2+} concentration is expressed as micromolar.

Statistical Analysis

Each set of experiments was repeated at least three times, and the results are presented as mean values $\pm$ SD. Statistical significance was assessed by Student's t test. The significance level was set at $P < 0.05$.

Results

Effect of Halothane on Ca^{2+} Signaling

The effect of halothane on basal $[Ca^{2+}]_i$ was assayed by adding 1.6 mM halothane to resting platelet suspensions. In 75% of the experiments performed (32 of 42), a small transient $[Ca^{2+}]_i$ increase of 17 $\pm$ 5 nM was detected (data not shown). This halothane-evoked Ca^{2+} increase was observed in the presence and in the absence of external Ca^{2+}, suggesting that the observed Ca^{2+} increase was related to a mobilization of Ca^{2+} from intracellular stores.

Figure 1 shows examples of fura-2 recordings obtained from experiments aimed at investigating the effect of halothane on the Ca^{2+} response induced by various platelet activators. Figure 2 summarizes the results of these experiments. In conditions in which platelets were exposed for 5 min to 1.6 mM halothane, there was a significant reduction of the initial U46619-evoked $[Ca^{2+}]_i$ increase from 227 $\pm$ 115 nM to 124 $\pm$ 89 nM (n = 10; fig. 2A). A significant decrease of the initial $[Ca^{2+}]_i$ peak from 286 $\pm$ 80 nM to 196 $\pm$ 67 nM (n = 6) was also observed after halothane treatment, when platelets were stimulated with 10 nM platelet-activating factor (fig. 2B). Similar results were obtained with thrombin (1 U/ml), with a reduction of the initial Ca^{2+} increase from 252 $\pm$ 128 to 149 $\pm$ 42 (n = 4), respectively (data not shown).

The agonist-evoked Ca^{2+} response in platelets is a biphasic process characterized by a release of Ca^{2+} from internal stores coupled to a Ca^{2+} influx from the external medium. To determine if halothane could selectively inhibit the release of Ca^{2+} from internal pools, platelets were stimulated in the absence of external Ca^{2+}. In these experiments, BAPTA, a Ca^{2+}-chelator, was added 50 s before exposure to U46619, as shown in figure 1C. Under these conditions, halothane caused a significant
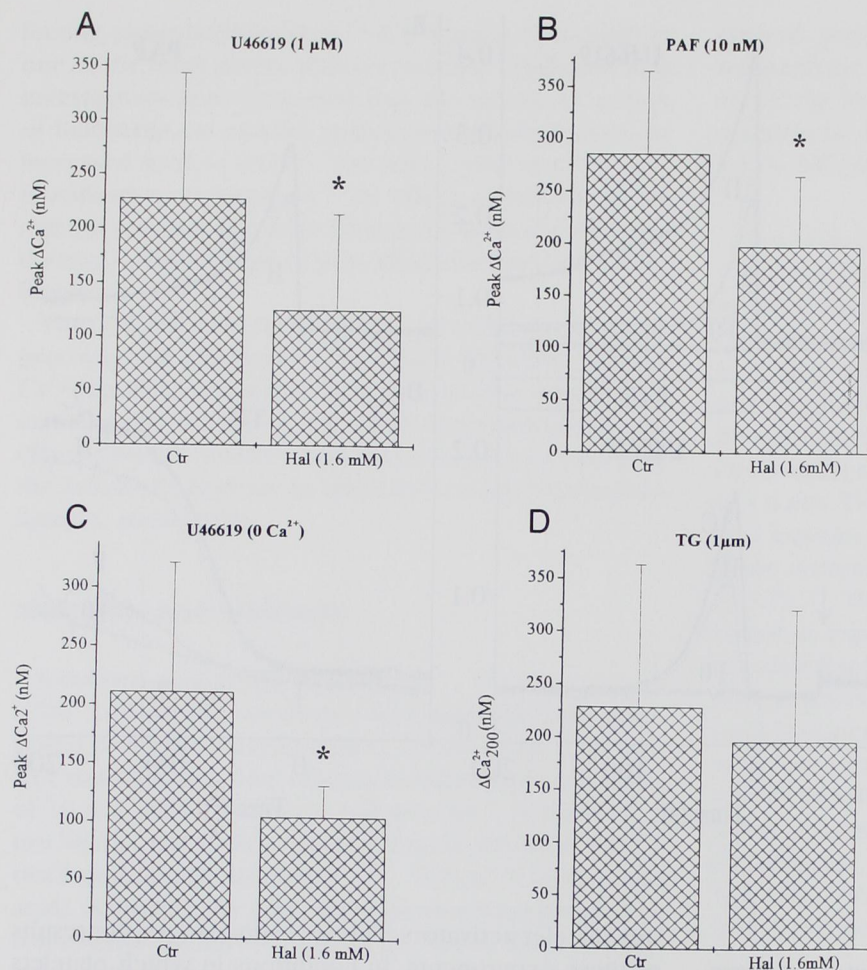
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Fig. 2. The effect of halothane on the Ca^{2+} response of platelets to different stimulating agents. The effect of halothane on the amplitude of the peak Ca^{2+} increase initiated by U46619 (1 μM; *A* and *C*) or platelet-activating factor (PAF; 10 nM; *B*), or the Ca^{2+} increase at 200 s triggered by thapsigargin (TG; 1 μM; *D*). A significant effect ($P < 0.05$) compared with control (Ctr) was observed in U46619 and platelet-activating factor-stimulated cells, but halothane did not affect the Ca^{2+} response when thapsigargin (TG) was used as stimulating agent. *$P < 0.05$. The analysis was based on n = 10 in *A* and n = 6 in *B, C,* and *D*.

decrease of the initial Ca^{2+} peak induced by U46619 from 210 $\pm$ 110 nM to 104 $\pm$ 25 nM (n = 7; fig. 2C), indicating an action of halothane on the mechanisms underlying the release of Ca^{2+} from internal pools. Similar results were obtained with platelet-activating factor and thrombin (data not shown).

To determine if the observed action of halothane on Ca^{2+} signaling requires the production of second messengers related to the phosphoinositide pathway, halothane experiments were performed in which platelets were stimulated with the sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase inhibitor, thapsigargin. Thapsigargin selectively block the internal Ca^{2+} sequestration process, leading to an uncompensated Ca^{2+} leak from internal stores. A mobilization of internal Ca^{2+} is generated under these conditions without producing the second messenger myo inositol-1,4,5 trisphosphate (InsP$_3$). In addition, this progressive and constant emptying of internal Ca^{2+} pools has been shown to trigger a Ca^{2+} entry

from the external medium, called capacitative Ca^{2+} influx.[7,19,20] The results in figures 1D and 2D show that halothane, at a supraclinical concentration of 1.6 mM, failed to significantly affect the $[Ca^{2+}]_i$ mobilization induced by thapsigargin, with a change from 227 $\pm$ 135 nM to 195 $\pm$ 124 nM (n = 7) of the Ca^{2+} level measured 200 s after thapsigargin application. These results suggest that stimulation of phospholipase C is essential for halothane to inhibit the mobilization of Ca^{2+} from internal Ca^{2+} pools and the related capacitative Ca^{2+} influx.

Effect of Forskolin on Ca^{2+} Signaling

Because halothane increased cAMP production in platelets, experiments were done to compare the action of forskolin and halothane on the Ca^{2+} response induced by U46619 or thapsigargin. Figures 1A and 1C show that a 5-min treatment with 1 μM forskolin completely abolished the Ca^{2+} increase triggered by U46619

in the presence (n = 4) or in the absence (n = 4) of external Ca^{2+}, respectively. However, in contrast to halothane, 1 μM forskolin succeeded also in inhibiting the Ca^{2+} response induced by thapsigargin (fig. 1D). The results in figure 1A indicate furthermore that the addition of 0.1 μM forskolin caused a 50% reduction of the mean U46619-evoked $[Ca^{2+}]_i$ increase with values of 250 ± 35 nM (n = 3) in control conditions and 123 ± 20 nM (n = 3) after forskolin treatment. An inhibitory effect of equivalent potency was observed after a 5-min exposure to 1.6 mM halothane (fig. 1A). However, forskolin at 0.1 μM appeared more potent than halothane in inhibiting the Ca^{2+} response initiated by thapsigargin, with a 42 ± 7% (n = 3) reduction of the internal Ca^{2+} level measured 200 s after thapsigargin application (fig. 1D). These results suggest that halothane and cAMP operate in different ways on Ca^{2+} signaling in platelets.

Effect of Halothane and Forskolin on the Capacitative Ca^{2+} Influx

The capacitative Ca^{2+} entry in platelets is characterized by an external Ca^{2+} influx that is intimately regulated by the emptying of internal Ca^{2+} stores.[7,19] To investigate the effect of halothane and forskolin on the capacitative Ca^{2+} entry in platelets, fura-2 experiments were performed in which thapsigargin was used to generate a release of Ca^{2+} from internal pools in the absence of $InsP_3$ production. In a first series of experiments, platelets were first treated for 5 min with either 1.6 mM halothane or forskolin (0.1 to 1 μM) before the addition of thapsigargin (1 μM) in the absence of external Ca^{2+}. The Ca^{2+} influx was initiated by the subsequent addition (100 s after thapsigargin) of 1 mM $CaCl_2$ to the external medium (fig. 3A). Pretreatment with 0.1 μM forskolin decreased by 50 ± 7% (n = 4) the internal Ca^{2+} level measured 100 s after the addition of external Ca^{2+}, whereas total inhibition of the Ca^{2+} response was observed in platelets exposed to 1 μM forskolin (data not shown). In contrast, under the same experimental conditions, halothane had a nonsignificant effect on the thapsigargin-evoked capacitative Ca^{2+} influx, with a reduction of the related Ca^{2+} increase of 10 ± 6% (n = 4).

In a second series of experiments, halothane and forskolin were added at the peak of the Ca^{2+} response after thapsigargin stimulation. The importance of the Ca^{2+} influx to the thapsigargin-induced Ca^{2+} increase was estimated in this case by chelating the external Ca^{2+} with BAPTA 50 s after thapsigargin exposure. Figure 3B shows that the addition of BAPTA resulted in a slow
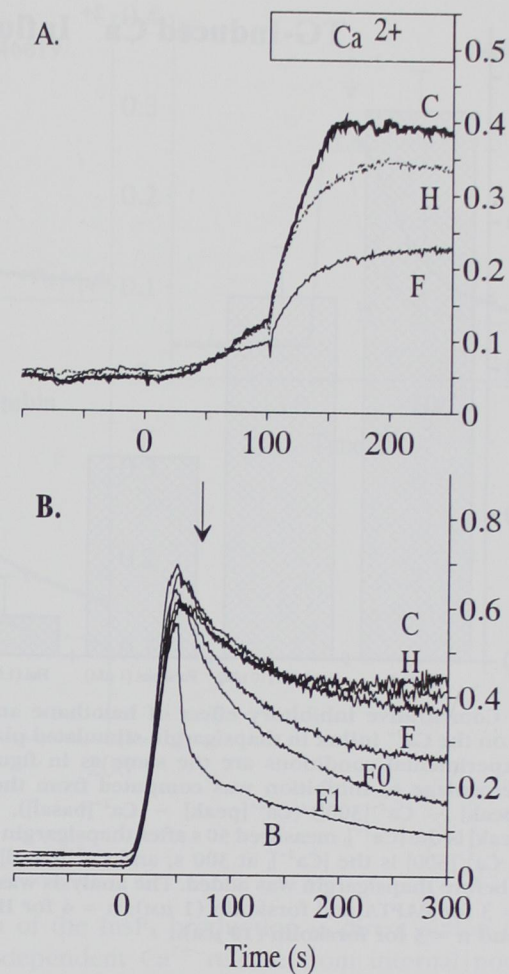

Fig. 3. Effect of halothane on the Ca^{2+} influx initiated by thapsigargin stimulation. (*A*) Ca^{2+} influx generated by Ca^{2+} readmission after withdrawal of Ca^{2+} from the external medium. Platelets were treated 5 min with either 1.6 mM halothane (H) or 0.1 μM forskolin (F) before fura-2 measurements in zero-external Ca^{2+} conditions. Thapsigargin (TG, 1 μM) was added 100 s before the readmission of Ca^{2+} in the external medium. Halothane had a minimal effect on the Ca^{2+} entry caused by Ca^{2+} readmission. (*B*) Acute addition of halothane or forskolin to thapsigargin-stimulated platelets. BAPTA (B), halothane (H), and forskolin (0.1 μM [F], 1 μM [F0], 10 μM [F1]) were added 50 s after thapsigargin (TG) stimulation. In the total absence of Ca^{2+} influx in BAPTA conditions, the internal Ca^{2+} level decayed to a low steady state value. In contrast, the addition of halothane had no effect on the Ca^{2+} level in thapsigargin (TG)-stimulated platelets. A dose–response inhibition of the Ca^{2+} entry was observed when platelets were exposed to forskolin at concentrations ranging from 0.1 to 10 μM. Control curves are labeled as C.

decay of the internal Ca^{2+} level to a lower steady-state value, indicating an absence of Ca^{2+} entry in external Ca^{2+} free conditions. Similarly, a dose-dependent de-

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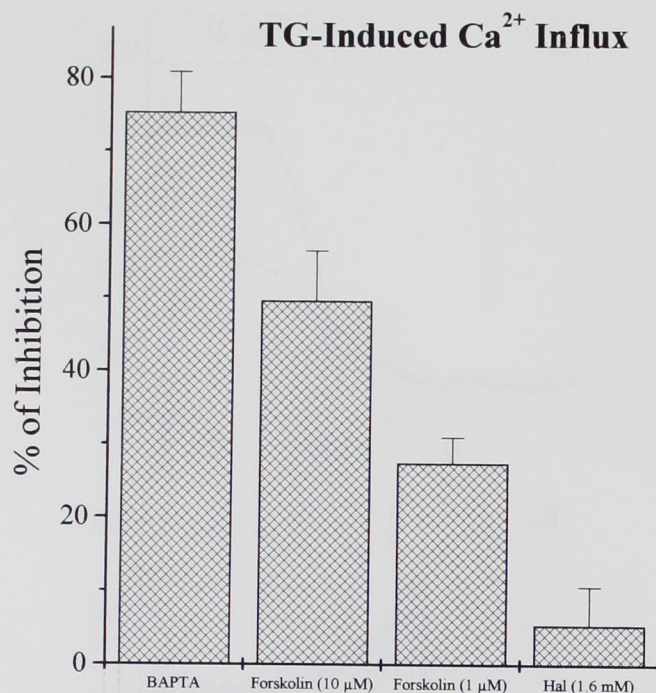## TG-Induced Ca^{2+} Influx

Fig. 4. Comparative inhibitory effect of halothane and forskolin on the Ca^{2+} influx in thapsigargin-stimulated platelets. The experimental conditions are the same as in figure 3B. The percentage of inhibition was computed from the ratio (Ca^{2+}[peak] − Ca^{2+}[300])/(Ca^{2+}[peak] − Ca^{2+}[basal]), where Ca^{2+}[peak] is the [Ca^{2+}]$_i$ measured 50 s after thapsigargin stimulation, Ca^{2+}[300] is the [Ca^{2+}]$_i$ at 300 s, and Ca^{2+}[basal] is the [Ca^{2+}]$_i$ before thapsigargin was added. The analysis was based on n = 3 for BAPTA and forskolin (1 μM); n = 4 for Hal (1.6 mM); and n = 5 for forskolin (10 μM).

crease of the fura-2 signal was observed after the addition of forskolin at concentrations ranging from 0.1 to 10 μM (fig. 3B). Figure 4 summarizes the results of these experiments. The presence of BAPTA caused a 75 ± 5% (n = 4) inhibition of the Ca^{2+} influx, compared with 49 ± 7% (n = 4) and 27 ± 4% (n = 4) with forskolin at 10 μM and 1 μM, respectively. As observed previously, halothane had no effect (5 ± 5%, n = 4) on thapsigargin-induced Ca^{2+} influx. These results provide additional evidence for a differential effect between halothane and forskolin on Ca^{2+} signaling machinery in platelets.

Acute Effect of Halothane on Ca^{2+} Signaling

The inhibition of the initial agonist-induced Ca^{2+} increase by halothane illustrated in figures 1A to 1C was measured after a 5-min pretreatment with halothane. Experiments were also performed in which halothane was added 30 s after the mobilization of Ca^{2+} in re-

sponse to several platelet activators. This protocol allows the internal stores to deplete rapidly and transitionally, with a maximal and sustained contribution of the Ca^{2+} influx process. The results presented in figure 5A to 5C indicate that the acute addition of halothane to U46619-, platelet-activating factor-, and thrombin-stimulated cells initiated a small increase in the rate at which the fura-2 signal decayed to a lower steady state value. These observations suggest that halothane can also rapidly affect the sustained agonist-mediated Ca^{2+} entry.

Effect of Halothane on Platelet Aggregation

Figures 6A and 6B show the effect of halothane and forskolin on platelet aggregation caused by U46619 and thapsigargin, respectively. The results indicate that halothane and forskolin acted differently, depending on the platelet activator used. A high concentration of forskolin (1 μM) completely inhibited platelet aggregation induced by either U46619 (n = 3) or thapsigargin (n = 4). In contrast, 1.6 mM halothane impaired the aggregation triggered by U46619 (n = 3) but failed to affect the thapsigargin-mediated aggregation process (n = 4).

Discussion

This study investigated the effect of halothane on platelet Ca^{2+} mobilization and aggregation. Our results essentially indicate that halothane inhibits the aggregation and initial Ca^{2+} release in platelets stimulated with agonists linked to the phosphatidylinositol pathway, but affect minimally the Ca^{2+} response and the aggregation process induced by thapsigargin.

Effect of Halothane on Agonist-evoked Ca^{2+} Responses

The results presented in figures 1A, 1B, 2A, and 2B indicate that halothane inhibits the internal Ca^{2+} increase induced by U46619 and platelet-activating factor. These results support the original observations of Kohro and Yamakage,[11] who reported a dose-dependent inhibition of the Ca^{2+} release induced by thrombin in human platelets. In their study, halothane was effective at clinically relevant concentrations (<1 mM), and complete inhibition of the Ca^{2+} increase triggered by thrombin was observed at 2 mM halothane, with half inhibition measured at 1 mM. There was no effect of isoflurane. In the current work, a near 50% inhibition was obtained at 1.6 mM halothane, depending on the agonist
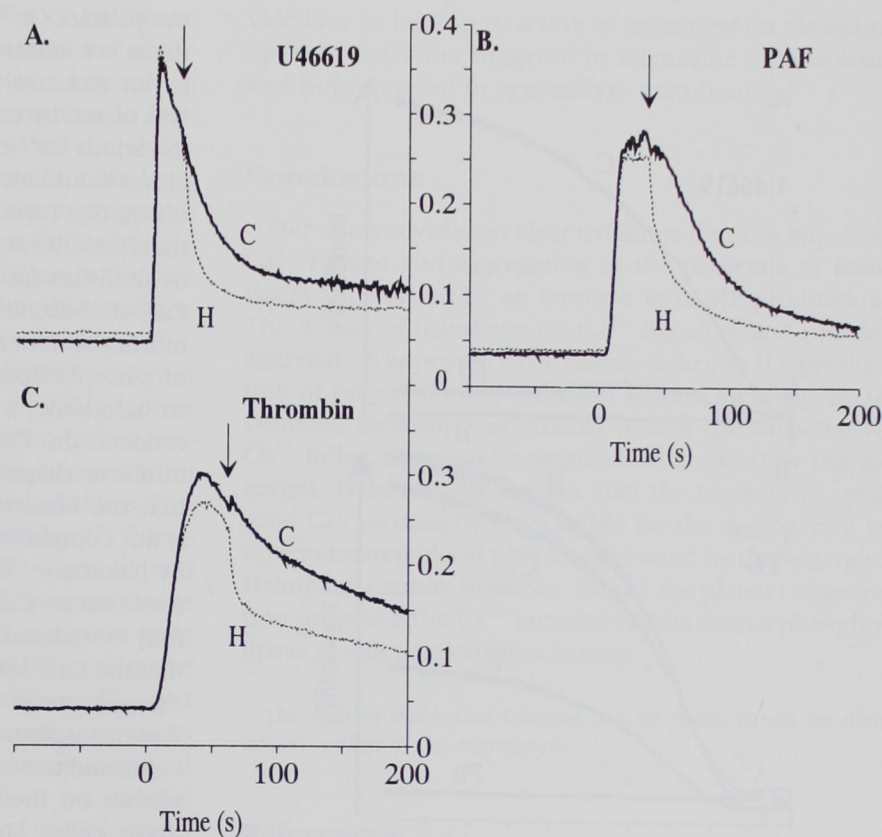
Fig. 5. The acute effect of halothane on the stimulated Ca^{2+} level induced by U46619, platelet-activating factor, and thrombin. Experiments were done in the presence of 1 mM external $CaCl_2$. Halothane (H, 1.6 mM) was added 30 s (*arrow*) after platelet stimulation with either 1 μM U46619 (*A*) or 10 nM platelet-activating factor (PAF) (*B*) and 60 s after stimulation with 1 U/ml thrombin (*C*). This protocol allows the fast depletion of internal Ca^{2+} stores, keeping an important contribution of the capacitative Ca^{2+} influx. Control curves are labeled as C. Cytosolic Ca^{2+} concentration is expressed as micromolar.

used as a stimulating agent. A higher halothane concentration in our case may be explained in part by differences in experimental conditions, such as the temperature (room temperature in the current study compared with 37°C in the work of Kohro and Yamakage[11]), because the same concentration of 1.6 mM halothane corresponds to a gaseous partial pressure of 5% at 37°C and 3% at 22°C.[16] Given the negative results obtained by Kohro and Yamakage,[11] we did not investigate isoflurane further in the current study.

The action of halothane may involve various specific mechanisms, including inhibition of the internal Ca^{2+} release from internal stores,[9,21] decrease of the Ca^{2+} influx,[10] or impairment of the Ca^{2+} extrusion processes.[22] In platelets, the Ca^{2+} response to U46619 stimulation results from a fast release of Ca^{2+} from internal stores and a sustained Ca^{2+} influx from the external medium.[7,23] The results in figures 1B and 2B provide clear evidence that halothane interferes strongly with the initial Ca^{2+} release process as already reported in some cell preparations.[9,21] This impaired Ca^{2+} release may result from several mechanisms, including an inhi-

bition of the $InsP_3$ production, a direct action on the $InsP_3$-dependent Ca^{2+} release from internal pools, or both. In this regard, evidence has already been provided for a reduced $InsP_3$ production in thrombin-stimulated human platelets exposed to halothane (2 mM).[11] In addition, the absence of a halothane-dependent inhibition of the Ca^{2+} increase initiated by thapsigargin argues for an effect of halothane on the production of second messengers linked to phospholipase C stimulation.

Further, when halothane was added after U46619 or thrombin or platelet-activating factor, there was a clear increase in the rate at which the $[Ca^{2+}]_i$ decayed to a lower steady state value (figs. 5A – C). Because Ca^{2+} influx is responsible for the sustained $[Ca^{2+}]_i$[7] increase observed under these conditions, halothane thus appears to inhibit Ca^{2+} influx, as reported in endothelial cells.[10] However, halothane had no effect on the capacitative Ca^{2+} influx induced by thapsigargin (figs. 3A and 3B) suggesting first that halothane does not significantly modify the Ca^{2+} extrusion process, and second that part of the Ca^{2+} influx in U46619-stimulated cells is mediated by a second messenger-dependent Ca^{2+} entry process
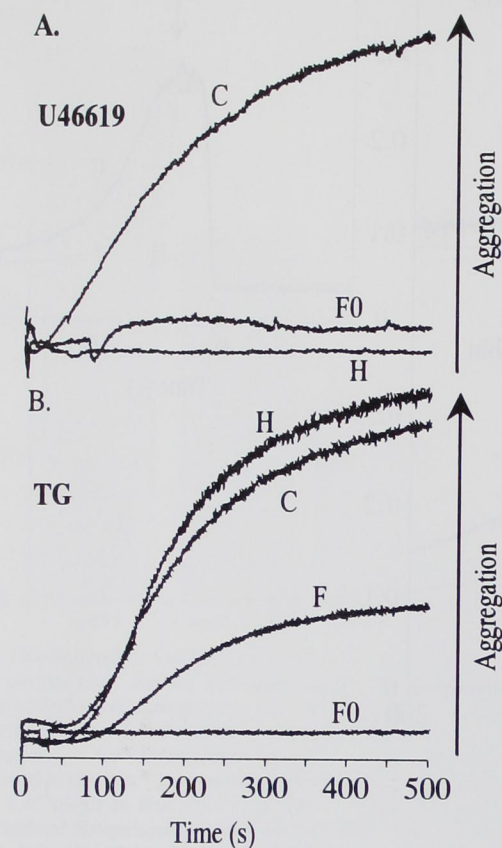
Fig. 6. The effect of halothane and forskolin on the aggregation induced by thapsigargin and U46619. Traces represent nephelometric measurements in which the transmittance is proportional to the extent of the aggregation. Platelets were treated for 5 min with either 1.6 mM halothane (H) or forskolin (0.1 μM [F] and 1 μM [F0]) before the addition at t = 0 s of 1 μM U46619 (A) or 1 μM thapsigargin (TG) (*B*).

distinct from the capacitative Ca^{2+} influx and that would be halothane sensitive. Kinetic experiments have already shown that Ca^{2+} influx precedes Ca^{2+} release,[24] suggesting that the capacitative Ca^{2+} influx is not exclusively responsible for the overall Ca^{2+} influx observed in U46619-stimulated platelets. Therefore, a direct correlation seems to exist between the production of second messengers, which participate in Ca^{2+} mobilization processes in platelets, and the ability of halothane to interfere with the Ca^{2+} signaling machinery.

The Effect of Forskolin on Ca^{2+} Signaling

Previous studies have shown that halothane increases cAMP production in platelets.[13] Because cAMP alters

the platelet Ca^{2+} signaling process,[6] the effects of halothane we observed could have been indirectly caused by an increase in cAMP production. This mechanism was tested by comparing the Ca^{2+} response of platelets to various Ca^{2+}-mobilizing agonists, after treatment with either halothane or forskolin, an agent that directly stimulates the formation of cAMP. Our results confirmed that forskolin at concentrations of 0.1 to 1 μM attenuates or abolishes (or both), in a dose-dependent manner, the Ca^{2+} mobilization initiated by U46619 (fig. 1A). Furthermore, the action of cAMP appeared to be independent of phospholipase C stimulation, because, in contrast to halothane, a pretreatment with forskolin markedly reduced the Ca^{2+} increase initiated by the Ca^{2+} pump inhibitor thapsigargin (fig. 1D). These results suggest that the observed effect of halothane on Ca^{2+} release is not compatible with a stimulation of cAMP formation by halothane. The mechanism underlying the action of forskolin on Ca^{2+} signaling in platelets is not, however, fully elucidated. A report by Koike *et al.*[25] has indicated that the Ca^{2+} influx induced in human platelets by thapsigargin could be partly suppressed by pretreatment with phosphatase inhibitors, such as okadaic acid, calyculin, and tautomycin, suggesting an effect of phosphorylation on the Ca^{2+} influx evoked by thapsigargin in these cells. These observations point to a different mechanism of action between forskolin and halothane on Ca^{2+} response.

Effect of Halothane and Forskolin on Platelet Aggregation

Because cAMP is recognized as a potent platelet aggregation inhibitor,[7,14] experiments were done to test the hypothesis that the effect of halothane on platelet aggregation[2-4] was mediated, at least in part, by cAMP. The results in figures 6A and B do not, however, support this hypothesis. For instance, 1.6 mM halothane completely inhibited the aggregation induced by U46619, an effect that could be mimicked by 1 μM forskolin (fig. 6A). The results in figure 6B indicate, however, that, in contrast to forskolin at 1 μM, halothane did not inhibit platelet aggregation initiated with thapsigargin, even when it was used at a supraclinical concentration of 1.6 mM. A model in which the action of halothane would be mediated exclusively by an increased cAMP production should have yielded identical results in both cases. Thus our observations correspond with the findings reported by Kohro and Yamakage[11] and do not support a cAMP-based effect of halothane on platelet aggregation (fig. 6A).

The mechanism underlying the action of halothane remains, however, ill defined. It is clear from our observations that cytosolic Ca^{2+} seems to play a key role in the effect of halothane on platelet aggregation. In fact, we observed a strong correlation between the ability of halothane to affect the Ca^{2+} response in stimulated platelets and its potency to impair platelet aggregation. For instance, halothane had no effect on Ca^{2+} mobilization and aggregation triggered by thapsigargin. In contrast, halothane significantly inhibited the platelet aggregation process in response to U46619 stimulation (fig. 6A), in agreement with the potent inhibitory action of halothane on the Ca^{2+} response induced by this agonist (figs. 1A and 1C). A similar relation between Ca^{2+} signaling and platelet aggregation can also be inferred from the results obtained from forskolin-treated platelets. Forskolin impaired the increase in intracellular Ca^{2+} triggered by both thapsigargin and U46619 (figs. 1A and 1D), supporting the platelet aggregation measurements presented in figures 6A and 6B. It is also apparent from these observations that the mechanisms underlying the action of halothane and forskolin on Ca^{2+} signaling and aggregation differ. In the case of halothane, our results point to a requirement for a phospholipase C-related second messenger formation for an effect on internal Ca^{2+} and platelet aggregation, as reported by Kohro and Yamakage.[11] Such a requirement was not observed in thapsigargin-stimulated platelets exposed to forskolin. In addition, our results showed that halothane does not affect the events leading to platelet aggregation downstream from the release of Ca^{2+} from internal pools and the initiation of a Ca^{2+} influx. Even when halothane was used at high concentrations (1.6 mM) and caused an inhibition of the Ca^{2+} response of platelets to Ca^{2+} mobilizing agonists, there was no effect of halothane on the thapsigargin-induced platelet aggregation, despite an increase of Ca^{2+} in this case. Thus halothane is likely to affect events before the Ca^{2+} release process. For instance, halothane may alter phospholipase C activation and decrease the production of both $InsP_3$ and diacylglycerol. This would in turn lead to a reduced $InsP_3$-mediated Ca^{2+} release while providing a mechanism whereby the effect of halothane on aggregation is mediated by a decreased protein kinase C (PKC) activity. In fact, PKC activation by phorbol esters has been shown to induce platelet aggregation through a Ca^{2+}-independent process.[26,27] It is thus possible that part of the action of halothane on platelet aggregation may be the result of an inhibition of the PKC-activation pathway. In support of this model, evidence has been provided for an inhibitory action of halothane on phospholipase C activation triggered by adenosine diphosphate and isoproterenol in erythrocyte membranes.[28]

Conclusions

Our study reveals no clear evidence that the impaired Ca^{2+} release and aggregation in the presence of halothane is mediated by an increase in cAMP production. The action of halothane on Ca^{2+} signaling and platelet aggregation appeared to be closely linked to the production of phospholipase C-related second messengers. In contrast, forskolin can directly inhibit the capacitative Ca^{2+} influx and platelet aggregation induced by thapsigargin. These results indicate that the increase in cytosolic Ca^{2+} is directly responsible for the aggregation in aspirin-treated blood platelets activated by thapsigargin. Halothane cannot, however, impair the platelet aggregation process if the Ca^{2+} increase results from a phospholipase C-independent mechanism.

References

1. Gibbs NM: The effect of anaesthetic agents on platelet function. Anesth Intens Care 1991; 19:495–505

2. Dalsgaard-Nielsen J, Risbo A, Simmelkjaer P, Gormsen J: Impaired platelet aggregation and increased bleeding time during general anaesthesia with halothane. Br J Anaesth 1981; 53:1039–42

3. Dalsgaard-Nielsen J, Gormsen J: Effects of halothane on platelet function. Thromb Haemost 1980; 44:143–5

4. Hofmann JG, Siedel U, Till U: Effect of halothane on metabolic ATP and induced aggregation of human blood platelets. Biomed Biochim Acta 1989; 48:337–41

5. Bertha BG, Folts JD, Nugent M, Rusy BF: Halothane, but not isoflurane or enflurane, protects against spontaneous and epinephrine-exacerbated acute thrombus formation in stenosed dog coronary arteries. ANESTHESIOLOGY 1989; 71:96–102

6. Siess W: Molecular mechanisms of platelet activation. Physiol Rev 1989; 69:158–78

7. Rink TJ, Sage SO: Calcium signaling in human platelets. Annu Rev Physiol 1990; 52:431–49

8. Freeman LC, Li Q: Effects of halothane on delayed after depolarization and calcium transients in dog ventricular myocytes exposed to isoproterenol. ANESTHESIOLOGY 1991; 74:146–54

9. Loeb AL, Longnecker DE, Williamson JR: Alteration of Ca^{2+} mobilization in endothelial cells by volatile anesthetics. Biochem Pharmacol 1993; 45:1137–42

10. Simoneau C, Thuringer D, Cai S, Garneau L, Blaise G, Sauvé R: Effects of halothane and isoflurane on bradykinin-induced Ca^{2+} influx in bovine aortic endothelial cells. ANESTHESIOLOGY 1996; 85:366–79

11. Kohro S, Yamakage M: Direct inhibitory mechanisms of halothane on human platelet aggregation. ANESTHESIOLOGY 1996; 85:96–106

12. Sprague DH, Yang JC, Ngai SH: Effects of isoflurane and halothane on contractility and the cyclic 3′,5′-adenosine monophosphate system in the rat aorta. ANESTHESIOLOGY 1974; 40:162–7

13. Walter F, Vulliemoz Y, Verosky M, Triner L: Effects of halothane on the cyclic 3′,5′-adenosine monophosphate enzyme system in human platelets. Anesth Analg 1980; 59:856–61

14. Haynes DH: Effects of cyclic nucleotides and protein kinases on platelet calcium homeostasis and mobilization. Platelets 1993; 4:231–42

15. Sage SO, Reast R, Rink TJ: ADP evokes biphasic Ca^{2+} influx in fura-2 loaded human platelets. Evidence for a Ca^{2+} entry regulated by intracellular stores. Biochem J 1990; 265:675–80

16. Franks NP, Lieb WR: Selective actions of volatile general anaesthetics at molecular and cellular levels. Br J Anaesth 1993; 71:65–76

17. Blaise G, To Q, Parent M, Lagarde B, Asenjo F, Sauvé R: Does halothane interfere with the release, action, or stability of endothelium-derived relaxing factor/nitric oxide? ANESTHESIOLOGY 1994; 80:417–26

18. Grynkiewicz G, Poenie M, Tsien RY: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985; 260:3440–50

19. Putney JW: Capacitative calcium entry revisited. Cell Calcium 1990; 11:611–24

20. Thastrup O, Linnebjerg H, Bjerrum PJ, Knudsen JB, Christensen SB: The inflammatory and tumor-promoting sesquiterpene lactone, thapsigargin, activates platelets by selective mobilization of calcium as shown by protein phosphorylations. Biochim Biophys Acta 1987; 927:65–73

21. Sill JC, Uhl C, Eskuri S, Van Dyke R, Tarara J: Halothane inhibits agonist-induced inositol phosphate and Ca^{2+} signaling in A7r5 cultured vascular smooth muscle cells. Mol Pharmacol 1991; 40:1006–13

22. Kosk-Kosicka D, Roszczynska G: Inhibition of plasma membrane Ca^{2+}-ATPase activity by volatile anesthetics. ANESTHESIOLOGY 1993; 79:774–80

23. Suganuma A, Nakasima S, Okano Y, Nozawa Y: Mass contents of inositol 1,4,5-triphosphate and 1,2-diacylglycerol in human platelets stimulated with a thromboxane analogue and thrombin. Thromb Haemost 1992; 68:341–5

24. Sage SO, Rink TJ: The kinetics of changes in intracellular calcium concentration in fura-2 loaded human platelets. J Biol Chem 1987; 262:16364–9

25. Koike Y, Ozaki Y, Qi R, Satoh K, Kurota K, Yatomi Y, Kume S: Phosphatase inhibitors suppress Ca^{2+} influx induced by receptor-mediated intracellular Ca^{2+} store depletion in human platelets. Cell Calcium 1994; 15:381–90

26. Zucker MB, Troll W, Belman S: The tumor-promotor phorbol ester (12-O-tetradecanoyl-phorbol-13-acetate), a potent aggregating agent for blood platelets. J Cell Biol 1974; 60:325–36

27. Rink TJ, Sanchez A, Hallam TJ: Diacylglycerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets. Nature 1983; 305:317–9

28. Rooney TA, Hager R, Stubbs C, Thomas AP: Halothane regulates G-protein-dependent phospholipase C activity in turkey erythrocyte membranes. J Biol Chem 1993; 268:15550–6