Anesthesiology
72:504–509, 1990

The Effects of Volatile Anesthetics on Ca^{++} Mobilization in Rat Hepatocytes

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This study provides direct evidence that in hepatocytes, intracellular Ca^{++} is released from internal stores by halothane, enflurane, and isoflurane. Hepatocytes isolated from rat livers were used fresh or treated with saponin and then incubated in $^{45}Ca^{++}$ media. The uptake of $^{45}Ca^{++}$ by hepatocytes was maximal following 13–16 min of incubation (untreated or saponin-treated) and the effects of various agents on the release of $^{45}Ca^{++}$ was studied following maximal loading. The agents used included halothane, enflurane, isoflurane, and several putative intracellular second messengers. The anesthetics, to various degrees, all stimulated a significant release of $^{45}Ca^{++}$ from internal stores at concentrations that were at or less than clinical concentrations. The release of intracellular $^{45}Ca^{++}$ by each of the anesthetic agents was dose-dependent with halothane and enflurane being equally potent at concentrations equivalent to 1 MAC exposure. The halothane-induced release was only somewhat suppressed by preincubation in either 2 mM $LaCL_3$ or 10 μM dantrolene, both suggested Ca^{++} channel blockers. Transient increases in intracellular Ca^{++} regulates a number of enzyme systems, including glycogenolysis, while prolonged elevation in Ca^{++} concentrations have been implicated in the mechanism of hepatotoxicity. (Key words: Anesthetics, volatile: halothane; enflurane; isoflurane. Animal: rat. Ions: calcium. Liver: isolated hepatocyte.)

IT IS KNOWN that the volatile anesthetics affect a variety of cellular functions that may or may not be related to the development of clinical anesthesia. The side effects of the agents may be of significant consequence on general cell function and therefore organ physiology and/or pathophysiology. In the study of the pharmacodynamics of the volatile anesthetics, it is of importance to determine the temporal sequence of effects. Hence, an increasing number of studies have been directed toward the interaction of anesthetics with those events that appear to be primary and that control the activity of a number of cellular biochemical events. For example, recent work has concentrated on the effects of volatile anesthetics on intracellular second messengers such as cyclic nucleotides[1–3] and free calcium (Ca^{++}).[4,5] While many of these studies have been directed towards the understanding of the effects of these anesthetics on events in nerves or muscles, these second messenger events may be common in all types of tissues.

Although cyclic nucleotide synthesis and Ca^{++} release is considered to be under the control of the adrenergic and/or cholinergic receptor systems,[6,7] the individual components of these systems may be affected by the volatile anesthetics differently. For example, numerous reports have appeared suggesting that halothane affects the activity of adenylate cyclase.[1,2] Halothane was shown to have a stimulatory effect on adenylate cyclase activity in some tissues (rat brain and liver),[2,8] but an antagonistic effect in others (dog and mouse myocardium).[1,9] Recently, strong evidence has emerged to suggest that halothane produces these effects by interacting in a specific manner with an important component of the receptor system: the guanine nucleotide binding protein (G-protein).[10,11]

In contrast to the effects on cyclic nucleotide synthesis, the effect of the volatile anesthetics on intracellular Ca^{++} homeostasis in hepatocytes has received little or no attention. It has been suggested that the hepatocyte uses changes in intracellular calcium concentration ($[Ca^{++}]_i$) as a principal second messenger, which may be reflected by the fact that the dominant receptor on the adult male hepatocyte is the α_1 adrenoceptor that signals Ca^{++}-dependent reactions.[12]

Currently, intense interest exists concerning the hypothesis of an altered $[Ca^{++}]_i$ homeostasis as a mechanism of cell death due to xenobiotics.[13] A recent study reported a delayed elevated total Ca^{++} in livers of guinea pigs exposed to halothane,[14] but this could be a secondary event consequent to cell injury. There have been no studies reporting an immediate Ca^{++} release in hepatocytes exposed to halothane. Since the $[Ca^{++}]_i$ is a result of multiple events, including intracellular release of Ca^{++}, the activity of the Ca^{++} pumps and the inflow of Ca^{++} from the medium, we have chosen to study the effects of the anesthetics on the release of Ca^{++} from intracellular stores. For these studies we have used rat hepatocyte suspensions

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Received from the Departments of Anesthesiology and Pharmacology, Mayo Clinic, Rochester, Minnesota. Accepted for publication October 8, 1989. Supported by U. S. Public Health Service Grants GM38033 (Van Dyke) and CA42286 (Powis) from the General Medical Sciences Institute and the National Cancer Institute, respectively. Presented at the Annual Meeting of the American Society of Anesthesiologists, New Orleans, Louisiana, October 1989.

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treated with saponin and report a dose-dependent release of Ca^{++} from intracellular stores by halothane, enflurane, and isoflurane.

Methods

This protocol was approved by the Institutional Animal Care and Use Committee. Livers from male Sprague-Dawley rats anesthetized with sodium thiopental were perfused through the portal vein with Krebs bicarbonate buffer gassed with 95% O_2, 5% CO_2 at a rate of 20 ml/min for 25 min. The livers were then perfused with a Krebs bicarbonate buffer containing 1.2 mM $CaCl_2$ and 50 mg/100 ml collagenase (Boehring, Mannheim, FRG) for 15 min at 37° C. The resulting cell suspension was filtered through a 250-μm nylon mesh and centrifuged at 200 rpm for 2 min (0–4° C; Beckman TJ–6); washed once with Krebs bicarbonate buffer containing 1.2 mM $CaCl_2$; and washed twice with Dulbecco's modified Eagle's medium (DMEM; Gibco Lab, Grand Island, NY). The cells were resuspended in DMEM at a concentration of 6×10^6 cells per ml at approximately 90% viability as measured by trypan blue exclusion, and kept at 4° C for no more than 3 h until use.

The suspended hepatocytes were washed twice by centrifugation at 800 g for 4 min with uptake buffer (140 mM KCl, 10 mM NaCl, 2.5 mM $MgCl_2$, and 10 mM HEPES/KOH, *p*H 7.0).[15] The saponin-treated cells were then incubated at 37° C for 20 min in uptake buffer containing 0.005% saponin (Sigma Chemical Co., St. Louis, MO). The saponin was then removed by rewashing the cells three times in saponin-free buffer. Cell concentration was determined on a hemocytometer and adjusted with buffer to obtain a final concentration of 5×10^6 cells per ml. The degree of permeability of the treated cells was checked by trypan blue penetration and was greater than 95% for each liver preparation studied (n = 5).

Hepatocyte suspensions (100 μl) were incubated with 200 μl of a medium containing 100 nM free $^{45}Ca^{++}$ at 37° C.[15] This medium consisted of uptake buffer containing 5% polyethylene gycol, 0.5 mM 2,4-dinitrophenol, 16 μM antimycin A (Sigma), 2 μg/ml oligomycin (Sigma), 0.375 mM EGTA, 1.5 mM ATP, 3 mM creatine phosphate (Sigma), 30 μg/ml creatine phosphokinase (Sigma), and 50 μM $CaCl_2$. Aliquots (100 μl) of loaded cells were removed at various times to determine the rate of uptake. The cells were collected on a vacuum filter system (VFM 1, Amicon, Danvers, MA) using glass fiber filters (Whatman GF/A, Maidstone, England). The cells were immediately washed four times with uptake buffer containing 1 mM $LaCl_3$. The filters were then removed and placed in scintillation vials. To each vial 0.5 ml of a tissue solubilizer (0.5 N quaternary ammonium hydroxide in toluene, Beckman, Fullerton, CA) was added. The vials were sealed with caps and placed in a drying oven (55° C) for 1 h. Finally, 10 ml of liquid scintillation cocktail (Aquasol, DuPont, Wilmington, DE) was added and radioactivity levels due to $^{45}Ca^{++}$ were determined.

When hepatocyte uptake of $^{45}Ca^{++}$ was considered stable, the effects of various agents on release was determined. First, an initial sample was removed from the incubation tube and filtered, 15 s later the various releasing agents were added, and exactly 2 min after the initial sample was filtered a second aliquot was processed. Thus, the amount of release was determined following incubation for 105 s at 37° C. The following agents were added as dilutions of a saturated aqueous buffer: halothane (Ayerst Laboratories, New York, NY), enflurane (Anaquest, Madison, WI), isoflurane (Anaquest), 1,4,5-inositol triphosphate (IP3) (Sigma), cis-6,9,12-octadecatrienoic acid (18:3, linolenic acid) (Sigma), and cis-5,8,11,14-eicosatetraenoic acid (20:4, arachidonic acid) (Sigma). The latter three agents were added to give final concentrations of 10 μM, 50 μM, and 50 μM, respectively. In all cases the volume of the addition was 10 μl. The final concentrations of each anesthetic agent in the incubation solutions were determined by gas chromatography.[16] The effects of dantrolene (Norwich Eaton, New York, NY), 1 and 10 μM, and lanthanum chloride ($LaCl_3$) (Sigma), 2 mM, on halothane-induced release were determined by incubation of the cells in these agents from time zero of the uptake procedure. For all preparations, control experiments were performed (*i.e.*, $^{45}Ca^{++}$ uptake was compared at 16 and 18 min).

The absolute amount of $^{45}Ca^{++}$ uptake by the hepatocytes was determined using the calculation described by Gill and Chuen.[17] All release data (*i.e.*, between 16 and 18 min) were calculated as percent difference from control. The effects of the blocking agents were compared to the released induced by halothane alone. The statistical significance of the data were determined using a one-sided ANOVA. Using this analysis, individual comparisons with a *P* value less than 0.01 was considered significant.

Results

The uptake of $^{45}Ca^{++}$ by the hepatocytes was maximal following 13–16 min of incubation. The uptake curves for both the untreated and saponin-treated cells had a similar time course (fig. 1). Thus, we determined the effects of various agents on the release of the $^{45}Ca^{++}$ that occurred between the 16- and 18-min periods. During this time there was minimal release and/or additional uptake of the $^{45}Ca^{++}$ by control incubation. In two of the

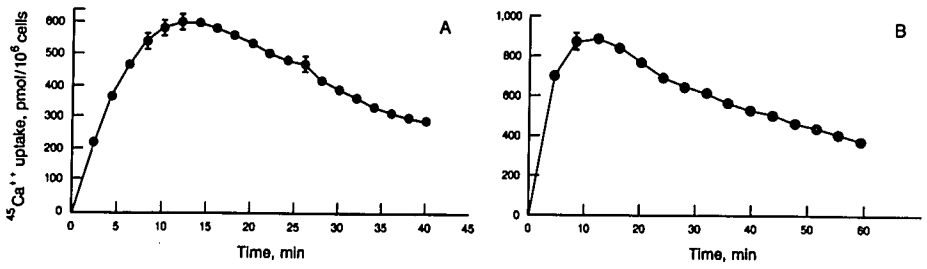

FIG. 1. The uptake of $^{45}Ca^{++}$ by both saponin-treated (*A*) and untreated (*B*) rat hepatocytes. These curves were obtained from different preparation of cells. The values were calculated from mean radioactivity levels ($\bar{X} \pm SD$) determined by liquid scintillation counting. The uptake in both experiments was maximal between 13–16 min.

four release experiments, the control measurements indicate further uptake of $^{45}Ca^{++}$; however, in each case this was minor (>4%). The average response between 16 and 18 min for the control experiments indicated a slight $^{45}Ca^{++}$ uptake: $-0.9 \pm 5.0\%$ (n = 13).

Halothane, enflurane, and isoflurane all induced reproducible, graded releases of the loaded $^{45}Ca^{++}$ (*i.e.*, three different hepatocyte preparations). Figure 2 shows the mean values of release and regression lines fit to the data. At 1 MAC (*in vivo* equivalent), halothane and enflurane are estimated to cause a similar release, whereas isoflurane would have little or no effect (fig. 2). All values were significantly different from control levels ($P < 0.001$) except for the release induced by the lower two concentrations of isoflurane ($P > 0.05$). Large releases of the $^{45}Ca^{++}$ were not induced by this agent until suprathera-peutic concentrations were achieved: $25.9 \pm 5.3\%$ at 6% isoflurane (n = 5).

Preincubation of the hepatocytes in dantrolene or $LaCl_3$ did not affect the uptake of $^{45}Ca^{++}$ (not shown). $LaCl_3$ significantly ($P < 0.001$), but not completely, blocked the $^{45}Ca^{++}$ release induced by the administration of 1% halothane (fig. 3). Dantrolene had little or no effect ($P > 0.05$). Figure 3 also shows that each of the putative second messengers (IP_3, linolenic acid, and arachidonic acid) induced significant releases of $^{45}Ca^{++}$ ($P < 0.001$). Finally, we noted that 1% halothane induced similar releases of $^{45}Ca^{++}$ in the untreated and saponin-treated hepatocytes (not significantly different, $P > 0.05$; fig. 3).

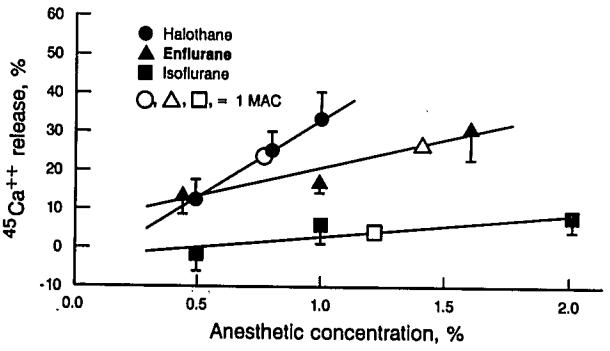

FIG. 2. The effects of graded concentrations of halothane, enflurane, and isoflurane on $^{45}Ca^{++}$ release. The mean values of anesthetic induced release ($\bar{X} \pm SD$) were plotted against the anesthetic concentration that were determined by gas chromatography as previously reported.[16] Regression lines were fit to the data. At 1 MAC (*in vivo* equivalent) halothane and enflurane were equally potent $^{45}Ca^{++}$-releasing agents. All mean values were significantly different from the control value ($P < 0.001$) except for the release induced by 1% ($P < 0.02$) and 0.45% ($P < 0.7$) isoflurane.

Discussion

A number of techniques are now available for the study of Ca^{++} homeostasis in intact living tissue. However, such studies are often complicated by experimental limitation. Thus we chose to simplify our approach and study only the release of Ca^{++} from endoplasmic reticulum. The exact mechanism of action of saponin on the hepatocyte is uncertain, but it is thought that the plasma membrane is made permeable by the removal of membrane-bound cholesterol.[18] Membrane systems such as those of endoplasmic reticulum or mitochondria have no cholesterol and therefore are considered to be unaffected by the sa-

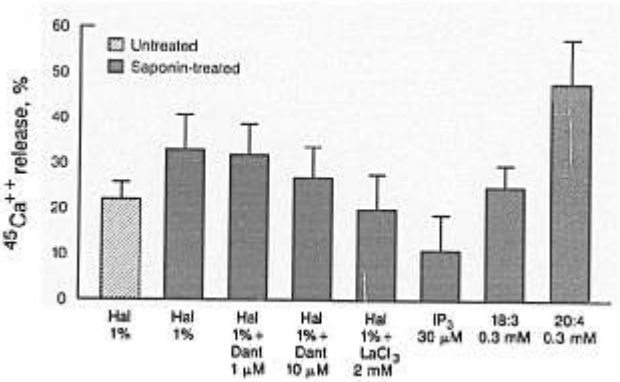

FIG. 3. The effects of blocking agents on 1% halothane-induced release of $^{45}Ca^{++}$ and release induced by several different putative second messengers. Mean values ($\bar{X} \pm SD$) derived from several experiments were plotted. The release of $^{45}Ca^{++}$ by 1% halothane was similar for untreated and saponin-treated cells. Preincubation in dantrolene or $LaCl_3$ did not affect the uptake of $^{45}Ca^{++}$ by the saponin-treated hepatocytes. However, $LaCL_3$ but not dantrolene significantly blocked the $^{45}Ca^{++}$ release induced by the administration of 1% halothane. IP_3, linolenic acid (C18:3), and arachidonic acid (C20:4) all induced significant releases of $^{45}Ca^{++}$ ($P < 0.001$).

ponin treatment. In agreement, there is ample evidence that the Ca^{++} uptake by sarcoplasmic reticulum in muscle is unaffected by saponin treatment,[19] as is the Ca^{++} uptake or release by microsomes or mitochondria from rat hepatocytes.[20,21] Hence, saponin treatment yields a model cell system in which the internal function of the hepatocyte is intact yet the cytosolic content can be readily controlled by the composition of the medium.

The data presented in this study clearly indicate that at anesthetic concentrations, halothane, enflurane, and isoflurane caused the release of Ca^{++} from internal Ca^{++} stores. Presumably, the major storage site was the endoplasmic reticulum and not mitochondria because these measurements were made in the presence of mitochondrial inhibitors.[21] While this distinction in intracellular source of Ca^{++} is not central to this discussion, it is consistent with results reported in other tissue that the endoplasmic reticulum has a much higher affinity and a more important role in controlling the intracellular-free [Ca^{++}] than the mitochondria.[20–22] Furthermore, halothane, enflurane, and isoflurane have been reported to inhibit the release of Ca^{++} from mitochondria.[23]

A second point to be derived from these data was that the release of Ca^{++} by the anesthetics was dose-dependent. The concentrations used in these studies were kept within the relatively narrow clinical range. However, it was apparent from figure 2 that the slope of the concentration-effect curve for halothane was statistically greater than for either enflurane or isoflurane. This suggests that halothane potentially might produce higher intracellular [Ca^{++}] within intact cells than either of the other two anesthetics. However, at 1 MAC (*in vivo* equivalent), halothane and enflurane produced similar releases and isoflurane had little or no effect.

The release of intracellular [Ca^{++}] may be part of the defense mechanism against xenobiotic exposure or other stressful conditions, since the Ca^{++} is suggested to stimulate a number of biochemical events to aid the cell in this effort.[24] On the other hand, events that elevate the intracellular [Ca^{++}] to high levels for prolonged periods have been associated with cell death.[25,26] Whether elevated Ca^{++} is the cause or effect of cell death has not been firmly established.[27] Nevertheless, it is interesting to speculate that this increase in Ca^{++} may be a factor in the anesthetic-induced hepatotoxicity seen in rats[28] and guinea pigs,[29] particularly in light of the recent report of massive accumulation of Ca^{++} in livers of guinea pigs 24 h after exposure to halothane.[14]

Of primary concern is the question, what is the difference between anesthetic-induced Ca^{++} release and hormone receptor-induced release? Perhaps very little, because both the receptors and anesthetics may interact with the same membrane component that is ultimately responsible for Ca^{++} release. Several reports have appeared recently suggesting that the volatile anesthetics can influence specific guanine nucleotide binding proteins (G-proteins) that control second messenger release.[10,11,30–32] While the G-protein associated with the α_1 adrenoceptor (the primary control of the signal transducing system associated with Ca^{++} mobilization) has not been characterized,[33] the possibility exists that anesthetics may interact with this protein as they have been proposed to interact with the G-proteins associated with β_2 adrenoceptor and muscarinic receptors.[10,11,30] Figure 3 contains evidence that IP_3 also stimulates the release of Ca^{++} from saponin-treated hepatocytes. IP_3 has been identified as the component of this system directly responsible for the release of Ca^{++} from the endoplasmic reticulum.[34] If the G-proteins are involved in this mechanism, the end point of their stimulation would be IP_3 synthesis and the data presented here simply shows that IP_3 was capable of stimulating the release. An alternative to the release of Ca^{++} by IP_3 is the direct release induced by opening different populations of ion channels. However, it is likely that one specific population of ion channels were not involved in this release since figure 3 also contains data indicating that $LaCl_3$, a specific ion channel blocker, had a minor effect on the release of Ca^{++} induced by halothane. It has been reported that dantrolene inhibited halothane-induced Ca^{++} release from the sarcoplasmic reticulum isolated from the skeletal muscle of pigs susceptible to malignant hyperthermia.[35,36] In addition, it was shown that dantrolene partially reversed the effects that halothane had on action potentials recorded from skeletal muscles isolated from susceptible animals.[37] Thus, we were interested if this agent would have any effect on the release of calcium induced by halothane in the Ca^{++} storage site of hepatocytes. It did not, thus one may conclude that: 1) the hepatic endoplasmic reticulum and the skeletal muscle sarcoplasmic reticulum have different populations of calcium channels and more than one may be altered by halothane; 2) dantrolene had a selective effect on a population of calcium channels that only exist in sarcoplasmic retilculum; 3) dantrolene has a different membrane selectivity for endoplasmic reticulum *versus* sarcoplasmic reticulum; or 4) that further studies are required. The release of intracellular Ca^{++} by linolenic and arachidonic acids has also been observed to occur in saponin-treated Swiss 3T3 fibroblasts.¶ In 3T3 cells, $LaCL_3$ completely blocks the release of Ca^{++} induced by arachidonic acid, but other blockers or known IP_3 inhibitors did not (*i.e.*, RO-31428, 10 μM ruthenium red or 100 μM heparin monosulfate). Thus, one may speculate that in the saponin-treated he-

¶ Submitted for presentation.

patocytes, Ca^{++} release may be induced *via* more than one pathway and/or population of channels.

Further studies will be required to identify the mechanism of release of Ca^{++} by the volatile anesthetics. While there is growing support for a direct effect of the volatile anesthetics on the G-proteins, evidence is also accumulating concerning the effect of these agents on various receptors as well as the effector portion of the receptor complexes. For example, parallel studies in this laboratory of such systems indicate that halothane, enflurane, and isoflurane alter the K_D and B_{max} of the α_1 adrenoceptor such that the receptor is converted to low-affinity type and the B_{max} is decreased.[38] This supports the evidence that the receptor portion of the receptor complex is not the site where the anesthetics are having their effect which induces Ca^{++} release. On the other hand, that the release is stimulated in the presence of a decreased receptor number and affinity offers interesting possibilities for direct effects on surface membrane components such as the G-protein as noted previously in this discussion.

While the majority of these studies have been conducted in saponin-treated hepatocytes, we also note that 1% halothane induced a similar release of $^{45}Ca^{++}$ in untreated cells (fig. 3). In addition, preliminary studies of Ca^{++} homeostasis in whole hepatocytes using the fluorescent dye fura-2 or the photoprotein aequorin support the fact that intracellular-released Ca^{++} is induced by anesthetics.[39] This may offer preliminary evidence that the release of Ca^{++} from intracellular stores exceeds the ability of the Ca^{++} pump to sequester Ca^{++}. The controversy regarding the question of whether elevated Ca^{++} is the cause or effect of hepatic necrosis will not be resolved by the studies described in this communication. However, it is interesting to note that the degree to which the various anesthetics induced Ca^{++} release is consistent with their known ability to induce hepatic necrosis (*i.e.*, halothane producing the greatest Ca^{++} release and hepatotoxicity). Nevertheless, this communication offers solid evidence that the volatile anesthetics release intracellular-stored Ca^{++} and do so in a dose-dependent manner.

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