Transient increases of intracellular Ca^{2+} induced by volatile anesthetics in rat hepatocytes

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Abstract — The affects of volatile anesthetics on mobilization of intracellular Ca^{2+} was monitored in primary cultures of rat hepatocytes using the fluorescent Ca^{2+} probe Fura–2. The use of Fura–2 was limited by several factors which complicated the quantitative analysis of the results, such as: (i) a high rate of dye leakage; (ii) changes in the redox state of the hepatocytes which interfered with the fluorescence produced by the dye at various excitation wavelengths; (iii) compartmentalization of the dye producing high local intracellular concentrations; and, of particular importance for this study, (iv) enhanced photobleaching of the dye in the presence of halothane. To aid in the interpretation of the Fura–2 data, the Ca^{2+}-sensitive photoprotein aequorin was also used to monitor changes in [Ca^{2+}]$_i$. The aequorin and Fura–2 techniques qualitatively yielded the same result, that the volatile anesthetic agents halothane, enflurane, and isoflurane induce an immediate and transient increase of [Ca^{2+}]$_i$. The durations of these transients were approximately between 5 and 10 min and were not related to any evident acute cell toxicity. The [Ca^{2+}]$_i$ increases induced by the volatile anesthetic agents were dose-dependent, with halothane the most potent. The exact mechanism governing these increases in [Ca^{2+}]$_i$ induced by these anesthetics in rat hepatocytes is unknown, but is likely to involve effects on both the cell surface membrane and endoplasmic reticulum components of the signal transducing system.

Intracellular free calcium concentration ([Ca^{2+}]$_i$) plays a vital role in the regulation of biochemical reactions essential, not only for normal cell function, but also for the cells' response to external conditions or to stimuli which may impose a stress on the cell (e.g. the presence of xenobiotics). Since the [Ca^{2+}]$_i$ is held constant by an elaborate system which is primarily membrane associated and volatile anesthetics have profound membrane effects, there is good reason to examine closely the influence these agents have on the [Ca^{2+}]$_i$. In a previous report from our laboratory we described experiments in which the effects of volatile anesthetics on intracellular membrane systems were studied. In those experiments contributions from effects on the plasma membrane were considered minimal: the volatile anesthetics stimulated the release of ^{45}Ca^{2+} from non-mitochondrial intracellular stores (saponin permeabilized cells were used) [1]. Although not shown in those studies, it has been established that volatile anesthetic agents decrease the rate of uptake of ^{45}Ca^{2+} into intracellular storage sites (unpublished data).

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A variety of techniques have been introduced to monitor changes in $[Ca^{2+}]_i$. Here we describe the use of two different Ca^{2+}-sensitive indicators in isolated rat hepatocytes. In the early 1980's, Tsien and colleagues first described the properties and advantages of fluorescent dyes as intracellular Ca^{2+} indicators [2–6]. Subsequently, numerous reports have described the use of one of the dyes, Fura–2, in a plethora of cell types including hepatocytes [7–10]. As experience and data on the use of this dye has accumulated and the limitations of its use have been documented, it has become apparent that the use of Fura–2 as an $[Ca^{2+}]_i$ indicator may not be appropriate for all cell types and experimental paradigms [11–16]. The other Ca^{2+} indicator which we have used is the Ca^{2+}-sensitive photoprotein, aequorin, which is isolated from the luminous hydromedusan, *Aequorea forskolea*. This indicator has also been used in a variety of cell types and several different techniques have been used to introduce it into the cytoplasm. These include microinjection, scrape loading and centrifugation [13, 17–24]. Aequorin is not toxic to most cells and once a cell is loaded with this biological indicator, the emitted luminescence can be used to estimate changes in $[Ca^{2+}]_i$.

The present study was designed to determine if increases in $[Ca^{2+}]_i$ can be induced in intact hepatocytes by various volatile anesthetic agents. Primary cultures of rat hepatocytes, loaded with either Fura–2 or aequorin, were exposed to several concentrations of halothane, isoflurane and enflurane. Both methods of estimating changes in $[Ca^{2+}]_i$ yielded qualitatively similar results, and establish that the volatile anesthetics increase $[Ca^{2+}]_i$, although only transiently in hepatocytes from normal, untreated, animals. Finally, we suggest that for rat hepatocytes the aequorin technique may have fewer limitations.

Materials and Methods

Preparation of hepatocytes

The protocol for these studies was approved by the Institutional Animal Care and Use Committee. Livers from male Sprague-Dawley rats, anesthetized with sodium thiopental, were removed and 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-micron 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, USA). The cells were resuspended in DMEM at a concentration of 6 x 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 before use [1].

Fura–2 measurements

Cells were plated on collagen (rat tail, Type 1, Sigma Chemical Co., St Louis, MO, USA) treated 25 mm circle cover glass (Fisher Scientific Co., Pitsburg, PN, USA) which was placed inside culture dishes (Primaria 3801, Falcon, Lincoln Park, NJ, USA). The plated cells were incubated in a DMEM solution containing 10% fetal bovine serum (Flow Laboratories, McLean, VA, USA), 5 units per ml penicillin-streptomycin solution (Gibco), 2 mM L-glutamine (Gibco) and 100 nM insulin (Sigma). Viability was measured at various times after cell isolation by trypan blue exclusion, and only those preparations with a viability of approximately 90% or greater were used.

Immediately prior to study, the cells were loaded with Fura–2/AM. These cell preparations were bathed for approximately 30–40 min at 37°C in a solution containing 10 μM Fura–2/AM (Molecular Probes, Eugene, OR, USA). These cells were then washed with either the standard Krebs buffer, or a modified low Ca^{2+} Krebs buffer in which the 1.2 mM $CaCl_2$ was omitted and quartz distilled water was used [18]. The low Ca^{2+} Krebs buffer was used to minimize changes in fluorescence from the extracellular media due to dye leakage [8]. The cells were then transferred to a specially designed chamber that was mounted on a temperature controlled microscope stage.

For excitation of fluorescence, the light from the 450 W Xenon lamp housed within a SPEX Fluorolog-2 Spectrofluorometer Imaging System (SPEX Industries Inc., Edison, NJ, USA) was directed into an inverted microscope (Diaphot, Nikon, Tokyo, Japan) equipped for fluorescence, photometry, and digital imaging. Light of various wavelengths from the Xenon lamp was eventually directed as parallel light into the objective (Fluor 40/1.30 oil, 160/0.17, Nikon) and to the preparation. The system, by utilizing a chopper wheel, could pass different beams of light through the specimen. This feature allowed for rapid switching between excitation light of 340 and 380 nm. Fluorescence emitted from the cells passed through an interference filter (510 nm center wavelength) to either a photomultiplier tube (SPEX) or to a SIT 66 camera (Dage-MTI, Inc, Michigan City, IN, USA). The relationship between the Fura-2 fluorescence ratio and $[Ca^{2+}]$ was calibrated using various Ca^{2+} buffering systems [5, 6, 20].

Aequorin measurements

The aequorin loading technique was a modification of methods previously used to incorporate aequorin into other cell types [21, 24]. A suspension of fresh hepatocytes, 5×10^6 cells/ml was washed three times by centrifugation at 800 g for 30 s in a 135 mM NaCl, 4 mM KCl, 11 mM glucose, and 0.5 mM potassium phosphate buffer, pH 7.4, at 4°C. For the third wash the medium also contained 1 mM EGTA. The hepatocytes were then suspended in 0.5 ml of solution containing 5×10^{-5} M aequorin (purchased from Dr J. Blinks, Mayo Clinic), 0.15 M KCl, and 50 mM HEPES, pH 7.4, and incubated with gentle shaking for 10 min at 4°C before being centrifuged at 800 g for 30 s. The cells were then suspended in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal calf serum and plated at 5–6 x 10^6 cells per 35 mm culture dish (Corning, Corning, NY, USA). The hepatocytes were plated down for 20–24 h at 37°C, gassed with humidified 95% air and 5% CO_2. Three hours prior to use the cells were incubated in DMEM without fetal calf serum.

An estimate of $[Ca^{+2}]_i$ was made by recording the light emission from serum-deprived aequorin-loaded cells. Culture dishes containing the aequorin-loaded hepatocytes were placed in a temperature-controlled holder at 37°C over a radio-frequency interference-shielded photomultiplier tube (9635QA, Thorn EMI Gencom, Fairfield, NJ, USA) in a light-tight chamber. The changes in current due to the emission of photons were converted to a voltage. All signals were then recorded via a strip chart recorder. At the end of each experiment the cells were lysed with 1 ml of 1% Triton X-100 containing 5 mM $CaCl_2$ and the total light signal was integrated. Changes in $[Ca^{2+}]_i$ were estimated using the methods previously reported [20, 22, 24]. In this method the integral of the light signal (L) obtained during the Triton X-100 exposure was multiplied by the peak-to-integral ratio for aequorin (approximately 2.6 at 37°C) to obtain a calculated peak intensity (L_{max}) that would have been observed had all the aequorin within the hepatocytes been discharged homogeneously and instantaneously. For each culture dish studied, a ratio of the resting or stimulated light emission to L_{max} was calculated and compared to an aequorin Ca^{2+} concentration-light emission curve constructed assuming an intracellular free Mg^{2+} concentration of 1 mM [22].

When aequorin luminescence was considered stable, the effect of various agents on release was determined. The following agents were added as dilutions of a saturated aqueous buffer: halothane (Ayerst Laboratories, New York, NY, USA), enflurane (Anaquest, Madison, WI, USA), isoflurane (Anaquest). The final concentrations of each anesthetic agent in the incubation solutions were determined by gas chromatography [25]. Carbon tetrachloride (Sigma), menadione (2-methyl-1,2-naphthoquinone; Aldrich, Milwaukee, WI, USA) and vasopressin (Sigma) were also used.

Statistical analysis

Statistical significance of the data was determined using an ANOVA: one factor completely randomized design. Using this analysis, for individual comparisons between means, the values of P are for a two-tailed test and a P-value < 0.05 was considered significant.

Results

Fura-2 experiments

Isolated rat hepatocytes were observed to be highly autofluorescent in the excitation wavelength range of Fura-2 (Fig. 1). As previously reported, the hepatocytes readily took up Fura-2/AM and converted it to Fura-2 [8]. However, the uptake of Fura-2 was not uniform and there was the formation of high local concentrations of Fura-2: 'hot spots' were observed visually. In addition, there was loss of cytoplasmic dye which was due to leakage, quenching, photobleaching and/or active extrusion of Fura-2 (Fig. 1).

Some degree of photobleaching of Fura-2 was observed for each preparation studied (e.g. Fig. 2) and, as previously reported, the rate of photobleaching was dependent on the light intensity [11–13]. We noted that the Fura-2 'hot spots' appeared to fade more rapidly than the dye located
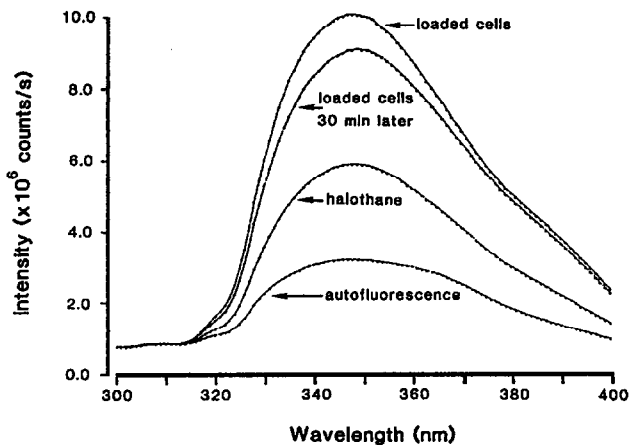
Table 1 Peak amplitudes and normalized areas of the Ca^{2+} transients estimated from the cells loaded with Fura-2

Agents	n	Resting $[Ca^{2+}]_i$ (μM)	Peak $[Ca^{2+}]_i$ (μM)
Halothane 0.5%	2	0.18 ± 0.07	0.23 ± 0.05
Halothane 1%	3	0.18 ± 0.05	0.28 ± 0.11
Halothane 2%	4	0.18 ± 0.05	0.28 ± 0.06
Halothane 4%	15	0.20 ± 0.22	0.32 ± 0.09
Isoflurane 4%	7	0.17 ± 0.04	0.27 ± 0.10
Enflurane 8%	9	0.18 ± 0.06	0.27 ± 0.07
Vasopressin (0.2 μM)	21	0.13 ± 0.04	0.24 ± 0.08
Ionomycin (50 μM)	6	0.16 ± 0.03	0.30 ± 0.06

Values are mean ± S.D. of n determinations. These experiments were performed using media which provided a low extracellular $[Ca^{2+}]$. The estimated peak amplitudes of the Ca^{2+} transients induced by each of the agents listed above were significantly different from control values in all cases ($P < 0.05$)

Fig. 1 The effects of time and halothane on the fluorescence excitation spectrum of rat hepatocytes loaded with Fura-2. The field of cells from which all spectra were recorded was the same. The initial autofluorescence was recorded and then the cells were incubated in 5×10^{-7} M Fura-2/AM for 1 h at 37°C. Subsequently, the extracellular dye was removed and an excitation spectrum immediately recorded. One half hour later a third excitation spectrum was obtained and a decrease of fluorescence noted, which was due to a loss of cytoplasmic dye or a change in the fluorescent properties of the dye. Finally, the cells were exposed to halothane (2%) for 5 min and subsequently a fourth spectrum was recorded. Low Ca^{2+} solutions were used for this experiment

in the remainder of the cell. Exposure of the hepatocytes to anesthetic concentrations of halothane greatly potentiated the photobleaching process and/or the leakage of intracellular Fura-2 (Figs 1–3). In the presence of halothane and light (including an excitation wavelength of 360 nm), the level of fluorescence which could be detected from the cells rapidly approached the level due to autofluorescence (Fig. 1). Light was required for the enhanced loss of fluorescence as indicated in Figure 3. Note that the initial level of fluorescence was also declining, which may be indicative of leakage. In this experiment the total population of hepatocytes was exposed to halothane but the rapid decrease in fluorescence only occurred in those cells exposed to the excitation light. We did not observe this same degree of enhanced photobleaching of fluorescence in those cells exposed to isoflurane or enflurane (results not shown). Superimposed on records showing the enhanced photobleaching transients were increases in $[Ca^{2+}]_i$ induced by halothane (Figs 2 & 3). However, the loss of fluorescence due to the halothane induced photobleaching made it difficult to evaluate absolute
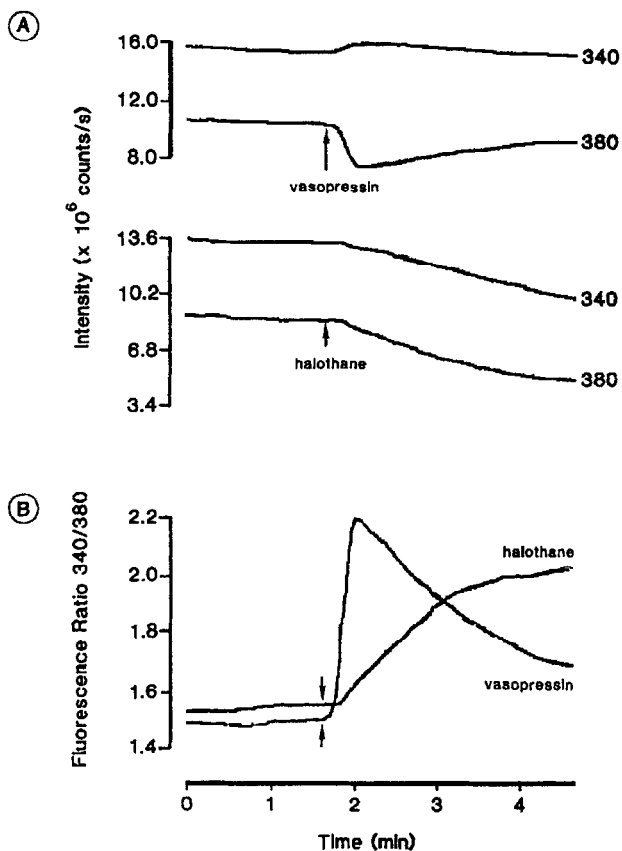
Fig. 2 Halothane causes an enhanced photobleaching of Fura–2 and a transient increase in hepatocyte $[Ca^{2+}]_i$

(A) The fluorescence emitted following excitation at either 340 or 380 nm: above the cells were exposed to 10^{-7} M vasopressin and below, to 2% halothane. Following excitation at either wavelength, the emitted fluorescence rapidly decreased only following the administration of 2% halothane

(B) The addition of either agent induced an increase in intracellular $[Ca^{2+}]$ as indicated by an increase in the 340/380 fluorescence ratio. The data shown here were obtained from successive cover slips of cells prepared from the same liver. Halothane and vasopressin were added to a low Ca^{2+} Krebs solution in these experiments

changes in $[Ca^{2+}]_i$.

The presence or absence of extracellular Ca^{2+} affected the magnitude or duration of the Ca^{2+} transients induced by the various anesthetic agents. Using Fura–2 the average resting $[Ca^{2+}]_i$ of the rat hepatocytes was estimated to be $0.130 \pm 0.060\ \mu M$ (n = 172 plates). Halothane caused a dose dependent change in the peak 340/380 fluorescence

ratio in the hepatocytes loaded with Fura–2 (Table 1). The peak increase in the estimated $[Ca^{2+}]_i$ occurred several minutes after the exposure to the halothane (e.g. Fig. 2). Similarly, enflurane and isoflurane also induced a dose dependent increase in $[Ca^{2+}]_i$. However, at similar anesthetic concentrations (e.g. 2%) halothane caused the largest increases and enflurane the smallest. We also observed that 0.1 μM vasopressin (Fig. 2) and ionomycin (50 μM) readily induced transient increases in $[Ca^{2+}]_i$ (see Table 1). However, it should be noted that although vasopressin induced a Ca^{2+} transient it did not cause enhanced photobleaching. In Figure 2, following the Ca^{2+} transient induced by vasopressin, the slight decrease in the level of fluorescence (excited at both wavelengths) was the same as that which would have been extrapolated due to normal rates of photobleaching.
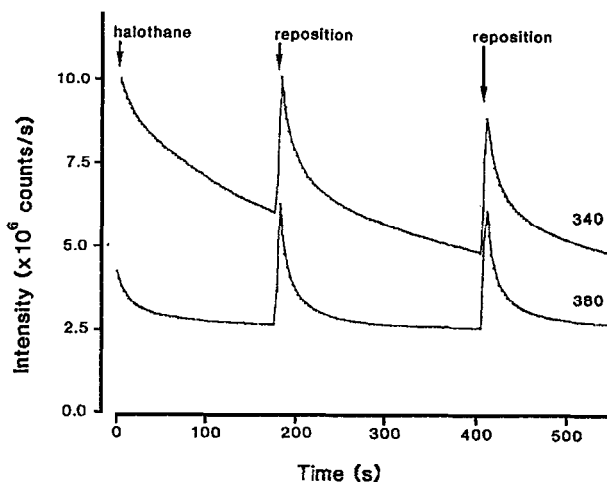

Fig. 3 Enhanced photobleaching following exposure to halothane. A monolayer of rat hepatocytes was exposed to approximately 2% halothane at time zero. The cells exposed to alternating 340 and 380 nm light rapidly lost their ability to fluoresce. When the field of cells was changed to cells previously not subjected to light, the initial level of detected fluorescence was high, but photobleaching rapidly followed. Note that the rate of decreased fluorescence in the latter two fields was similar following excitation at either wavelength. Hence, it appears that both the Ca^{2+} bound and unbound forms of the dye are equally sensitive to the enhanced photobleaching which was detected following the administration of halothane (to a low Ca^{2+} solution)

Aequorin experiments

The resting $[Ca^{2+}]_i$ estimated using aequorin in the hepatocytes prior to the administration of any drug was 0.162 ± 0.056 µM (n = 64 plates). Halothane, isoflurane and enflurane each induced an increase in $[Ca^{2+}]_i$ as detected by an increase in aequorin luminescence. The peak increase in the luminescence occurred several minutes after the exposure of the cells to the anesthetics (Fig. 4). The potency of the individual anesthetics was different from that observed previously in $^{45}Ca^{2+}$ experiments

Vasopressin

Fig. 5 Vasopressin (0.2 µM) induced a transient increase in luminescence. The hepatocytes were loaded with aequorin by a centrifugation method

(i.e. at similar anesthetic concentration, halothane induced the largest change and enflurane the smallest) [1].   We also noted that 0.1 µM vasopressin induced a large transient increase in luminescence (i.e. $[Ca^{2+}]_i$; Table 2 and Fig. 5).

The Ca^{2+} transients induced by the anesthetics were quite different from the changes in aequorin luminescence recorded following exposure of hepatocytes to either menadione or carbon tetrachloride (Fig. 6). These latter agents, in the concentrations used, are considered to be toxic.

In one experiment the same batch of aequorin loaded cells was studied both 24 and 48 h after loading. After 48 h, only halothane induced a transient change in $[Ca^{2+}]_i$; neither isoflurane (>12%) nor enflurane (>15%) had any effect (Fig. 7). It should be noted that the potentially reactive concentration of aequorin in the hepatocytes was 5 times lower following 48 h of incubation versus 24 h.
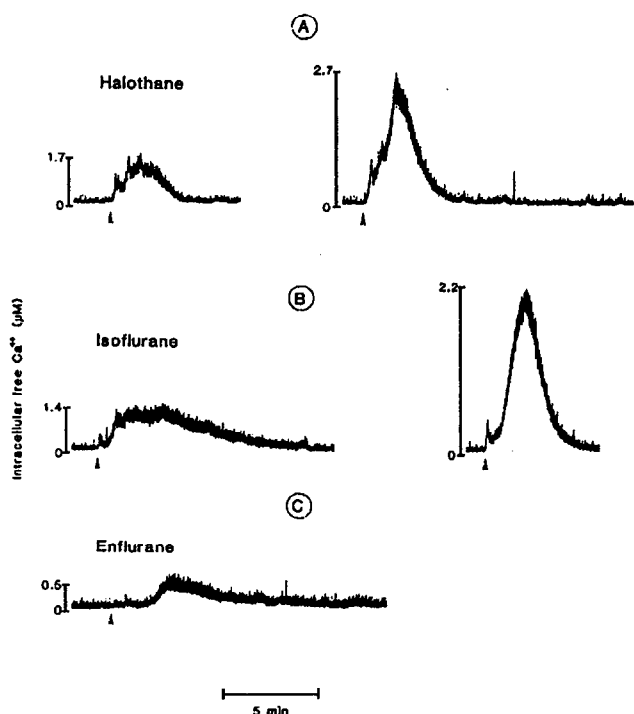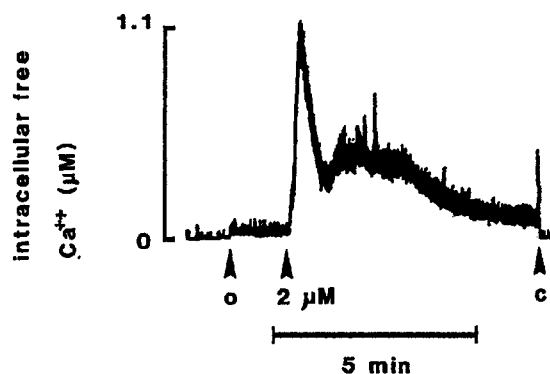
Fig. 4 Transient increases of $[Ca^{2+}]_i$ induced by exposing hepatocytes loaded with aequorin to halothane, isoflurane or enflurane.   The cells were loaded with aequorin using a centrifugation method. Following the loading procedure the cells were plated in culture dishes for at least 20 h. The anesthetic agents were administered by adding dilutions of saturated solutions. The data represent the average response of several millions cells, prepared from the same liver to the various anesthetic agents. In (A) the halothane concentrations were 1.5% and 3% (left to right); in (B) the isoflurane concentrations were 2% and 5%; and in (C) the enflurane concentration was approximately 10%.   The arrows indicate the time the administration began; note the delayed response to the large dose of enflurane (C)

Discussion

The effects of the volatile anesthetics on calcium mobilization

A variety of non-physiological conditions can increase intracellular Ca^{2+} beyond the normal range.

Table 2 Peak amplitudes and normalized areas of the Ca^{2+} transients recorded from cells loaded with aequorin

Agents	n	Resting $[Ca^{2+}]_i$ (μM)	Peak $[Ca^{2+}]_i$ (μM)	Normalized area ($mV \cdot s/L_{max}$)
Halothane 1%	4	0.16 ± 0.01	0.93 ± 0.04	6.2 ± 1.2
Halothane 2%	6	0.18 ± 0.07	0.84 ± 0.46	4.4 ± 4.1
Halothane 4%	14	0.17 ± 0.06	2.20 ± 1.48	72.1 ± 99.2
Isoflurane 8%	4	0.23 ± 0.10	2.00 ± 0.48	21.6 ± 23.0
Enflurane 15%	6	0.15 ± 0.06	1.96 ± 1.56	21.1 ± 31.7
Vasopressin (0.2 μM)	6	0.16 ± 0.03	0.76 ± 0.22	2.1 ± 3.8

The peak amplitudes and areas of the $[Ca^{2+}]_i$ transients were obtained by digital analysis of records of the original strip chart data. The peak $[Ca^{2+}]_i$ were estimated using the methods previously reported [22, 24]. The areas of the $[Ca^2]_i$ transients ($mV \cdot s$) were normalized by dividing by the L_{max} determined in each experiment. Values are mean ± S.D. of n determinations. The estimated peak amplitudes of the Ca^{2+} transients induced by each of the agents listed above were significantly different from control values in all cases ($P < 0.05$)

Menadione

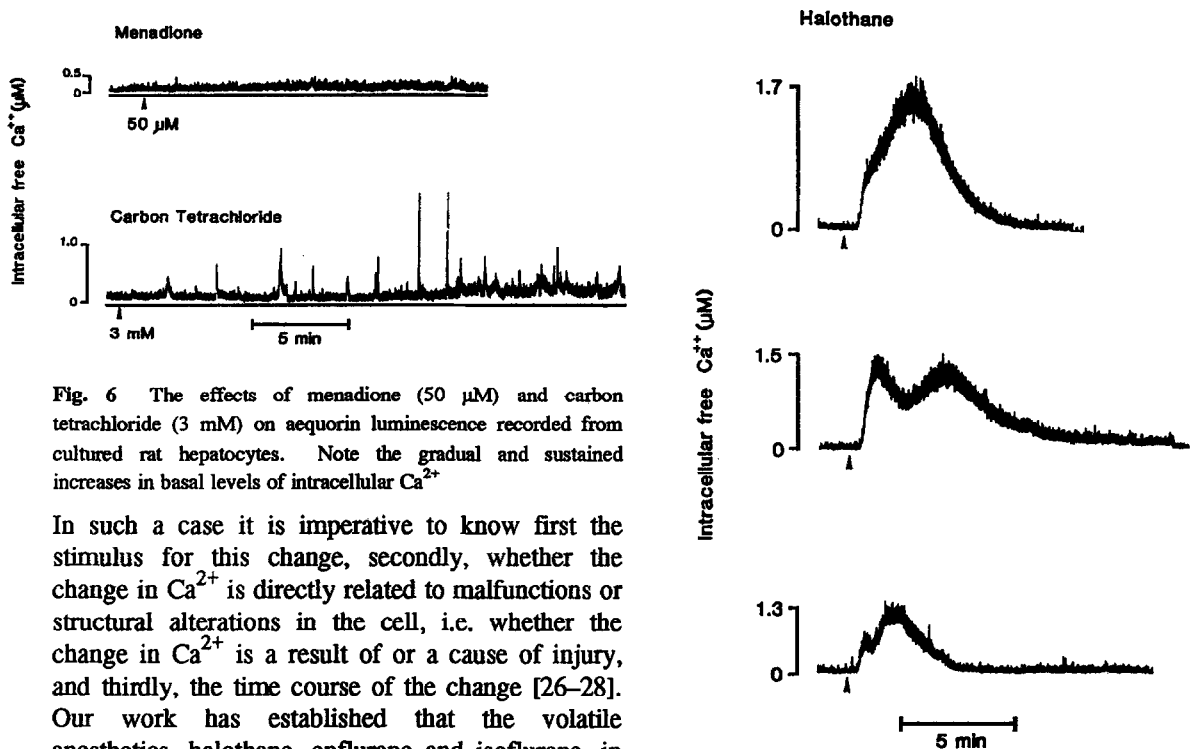**Carbon Tetrachloride**

Intracellular free Ca^{++} (μM)

Fig. 6 The effects of menadione (50 μM) and carbon tetrachloride (3 mM) on aequorin luminescence recorded from cultured rat hepatocytes. Note the gradual and sustained increases in basal levels of intracellular Ca^{2+}

In such a case it is imperative to know first the stimulus for this change, secondly, whether the change in Ca^{2+} is directly related to malfunctions or structural alterations in the cell, i.e. whether the change in Ca^{2+} is a result of or a cause of injury, and thirdly, the time course of the change [26–28]. Our work has established that the volatile anesthetics, halothane, enflurane and isoflurane, in clinical or slightly higher concentrations, can cause the release of Ca^{2+} from intracellular stores within seconds of their administration. This has been shown in the present study using Fura-2 and aequorin, and previously [1], with $^{45}Ca^{2+}$: i.e. a transient increase in $[Ca^{2+}]_i$ which a healthy, unstressed cell is able to restore to normal levels.

Halothane

Intracellular free Ca^{++} (μM)

Fig. 7 The effects of graded halothane concentrations on the $[Ca^{2+}]_i$ of hepatocytes which were incubated for 48 h after loading with aequorin. From top to bottom trace, the estimated halothane bath concentrations were 3, 1.5 and 1%

These transient increases in $[Ca^{2+}]_i$ were similar in amplitude, but slightly longer in duration, to those produced by the neurohormone, vasopressin.

However, two recent reports offer the possibility that in terms of toxicity of anesthetic agents, the initial response may somehow lead to greater and more sustained Ca^{2+} release. Farrell et al. have found an alteration in Ca^{2+} homeostasis in livers of guinea pigs exposed to halothane 24 h after exposure [29]. The total hepatic Ca^{2+} increased by 10-fold and the severity of liver necrosis was proportional to this increase. Nicotera et al. reported the disruption of Ca^{2+} homeostasis in hepatocytes by a metabolite of acetaminophen, N-acetyl-p-benzoquinone-imine (NAPQI) [30]. Previously it had been reported that acetaminophen in high doses caused a gradual increase in liver Ca^{2+} beginning 2 h after administration and reaching a maximum 24 h after treatment [31]. Other recent evidence that intracellular Ca^{2+} sequestration is a mediator of toxic events and leads to hepatic cell death have been reported [32–34]. Agents implicated have included CCl_4, chlordecone, and 1,1-dichloroethylene. Our data support these findings, with the observation that menadione and CCl_4 both induced slow but progressively increasing elevations of $[Ca^{2+}]_i$. Nevertheless, it remains unclear what physiological or environmental conditions are necessary to cause the xenobiotics to overwhelm the normal defense mechanisms and cause a sustained elevation of $[Ca^{2+}]_i$.

Our studies utilized only hepatocytes from animals which were considered normal (i.e. untreated) and only transient increases in $[Ca^{2+}]_i$ induced by the xenobiotics were observed. Prolonged elevations in $[Ca^{2+}]_i$ could be explained by: (i) unrestrained influx of extracellular Ca^{2+}; (ii) excessive release of Ca^{2+} from intracellular stores; (iii) loss of ability to pump Ca^{2+} out of the cell or back into storage areas; or (iv) a combination of increased release and decreased Ca^{2+} pump activity. Previous studies in our laboratory have indicated that, at least in the case of halothane, the uptake of Ca^{2+} is not stimulated by the anesthetic (unpublished data). At this time, we presume that the other agents would have a similar effect, although this requires study. Since the Ca^{2+} pump requires high energy input (ATP), we suspect that there is a strong possibility that in order to produce a sustained elevation in $[Ca^{2+}]_i$ there must be additional conditions imposed on the cell to interfere

with the supply of energy, thereby exacerbating the transient increases in $[Ca^{2+}]_i$ we observed into a much greater, perhaps lethal, effect. In support of this contention, previous reports have implicated the requirement of hypoxia and/or fasting conditions in order to produce hepatotoxicity in vivo by volatile anesthetic agents [35, 36].

A comparison between the Fura–2 and aequorin methods

Qualitatively the two methods used to monitor $[Ca^{2+}]_i$ provided consistent results, but quantitatively subtle differences were noted. For example, the levels of resting $[Ca^{2+}]_i$ were estimated to be slightly lower using the Fura–2 method. In addition, the durations of the transients induced by the various anesthetic agents were somewhat shorter within the aequorin loaded cells. This relationship is similar to that reported for changes in $[Ca^{2+}]_i$ induced within human neutrophils by various agents monitored using either the Fura–2 or aequorin methods [37]. Such differences may be explained by slight differences in our experimental designs, different kinetic reaction rates between the two indicators, by different localizations of the indicators within the cells, or perhaps by different buffering capacities of these indicators. In addition, the use of Fura–2 to monitor changes of $[Ca^{2+}]_i$ induced by halothane is complicated by the fact that, under these circumstances, enhanced photobleaching of the dye occurs. The lower peak amplitudes of the Ca^{2+} transients recorded in the Fura–2 experiments compared to the aequorin experiments were the result of a lower extracellular $[Ca^{2+}]$; in experiments where aequorin was used, the transients induced by these anesthetic agents are suppressed, but not eliminated, by the presence of 0.5 mM EGTA (unpublished data).

Enhanced photobleaching induced by halothane

A decrease in fluorescence from a cell preparation loaded with Fura–2 might result from either: (i) photobleaching; (ii) quenching of fluorescence; and/or (iii) enhanced loss of dye by cell membrane leakage [11–13]. It has previously been suggested that halothane may enhance the photobleaching of

Fura–2 [38]. We now show that the rapid decrease of Fura–2 fluorescence following the administration of halothane required light. Others have suggested that Fura–2 photobleaching was not equivalent to simply decreasing the intracellular concentration of the dye [39]. If this were the case, then the effect to some degree could be corrected by the ratio method of analysis. The problem may be related to the production of a photobleaching decay product(s) with different fluorescent characteristics and/or Ca^{2+} affinity than the parent compound. There is considerable reason to suspect that the multiple Fura–2 metabolites reported by Oakes et al. may in part be the result of photobleaching and not entirely related to metabolism [8].

Halothane is an extremely electrophilic compound which has been shown to undergo reductive as well as oxidative metabolism [25, 40]. The first step of either metabolic pathway is the release of bromide from the halothane molecule, a consequence of the addition of an electron. This results in the formation of an intermediate radical, which in the presence of oxygen is quickly oxidized, but under hypoxic conditions the radical will bind to adjacent molecules, e.g. unsaturated fatty acids [25, 40]. While no direct evidence has been obtained which supports this type of reaction, we speculate that free radical products contribute to Fura–2 breakdown. In support of this mechanism, perhaps, is the fact that neither isoflurane nor enflurane enhance photobleaching; these two agents produce the same effect on membrane stability as halothane, but do not form radical intermediates. We anticipate a number of other drugs or chemicals will contribute to photobleaching (as does halothane), but only if they are strong electrophiles which are associated with free radical formation.

In conclusion, by monitoring either changes in Fura–2 fluorescence or changes in aequorin luminescence, we noted that: (i) transient increases in $[Ca^{2+}]_i$ were induced by the volatile anesthetics halothane, isoflurane and enflurane and that (ii) the recorded transients were similar to those induced by vasopressin and they were all considered to be non-toxic responses. Extracellular Ca^{2+} enhanced, but was not required for, the induced changes in intracellular Ca^{2+}, which may indicate that one source of Ca^{2+} was from internal stores. Finally,

the use of aequorin luminescence to monitor changes in $[Ca^{2+}]_i$ induced by halothane within hepatocytes may have fewer limitations than the use of Fura–2; for example, we noted an enhanced photobleaching of Fura–2 fluorescence when hepatocytes were exposed to halothane.

Acknowledgements

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