

## Enhanced Mobilization of Intracellular $\text{Ca}^{2+}$ Induced by Halothane in Hepatocytes Isolated from Swine Susceptible to Malignant Hyperthermia

Paul A. Iaizzo, Ph.D.,\* Markus J. Seewald, Ph.D.,† Richard Olsen, M.S.,‡ Denise J. Wedel, M.D.,\* Dennis E. Chapman, Ph.D.,† Margareta Berggren,§ Hans M. Eichinger, Ph.D.,§ Garth Powis, D.Phil.¶

Halothane, in a dose-dependent manner, induced the release of intracellular  $\text{Ca}^{2+}$  in hepatocytes prepared from swine. The magnitude of the release induced by halothane was greater for hepatocytes prepared from animals susceptible to malignant hyperthermia (MH) than for those from normal swine. Two different methods were used to ascertain the release of  $\text{Ca}^{2+}$  induced by halothane: 1) the release of  $^{45}\text{Ca}^{2+}$  from nonmitochondrial stores of saponin-permeabilized hepatocytes was measured; and 2) changes in luminescence from intact hepatocytes loaded with the  $\text{Ca}^{2+}$ -sensitive photoprotein aequorin were recorded. It was also observed that, although 1,4,5-inositol trisphosphate ( $\text{IP}_3$ ), guanosine-5-triphosphate, and arachidonic acid all induced a significant release of  $^{45}\text{Ca}^{2+}$  from permeabilized swine hepatocytes, only the quantities of  $^{45}\text{Ca}^{2+}$  released by  $\text{IP}_3$  were significantly greater for the hepatocytes prepared from the animals susceptible to MH. These data indicate an abnormal  $\text{Ca}^{2+}$  homeostasis in hepatocytes isolated from swine susceptible to MH, which supports the hypothesis that membrane systems from multiple organs may be affected in this genetic disorder. (Key words: Anesthetic, volatile: halothane. Animal: swine. Endoplasmic reticulum:  $^{45}\text{Ca}^{2+}$  uptake and release. Genetic disorder: malignant hyperthermia. Intracellular  $[\text{Ca}^{2+}]$ : aequorin luminescence. Liver: isolated hepatocytes.)

EPISODES OF MALIGNANT HYPERTHERMIA (MH) can be initiated in genetically predisposed humans and swine by administration of volatile anesthetics such as halothane. These life-threatening episodes are primarily characterized by generalized muscle spasms (*i.e.*, contractures), tachycardia, increased core temperature, myoglobinuria, increased creatine phosphokinase levels, and/or cardiac arrest.<sup>1</sup> Numerous cellular membrane processes have been reported to be abnormally regulated in humans and swine susceptible to MH.<sup>1-19</sup> It is believed that abnormal regulation of the intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) within skeletal muscle, leading to contractures and eventual hypermetabolism, is the process of primary signifi-

cance in episodes of MH induced by volatile anesthetics.<sup>4,6,9,11,20-22</sup> The exact mechanism by which various so-called triggering anesthetics increase the release of  $\text{Ca}^{2+}$  has not been established, although the following have been reported: 1) the  $\text{Ca}^{2+}$ -release channel (*i.e.* ryanodine receptor) of the sarcoplasmic reticulum is defective in MH<sup>9,11,23-28</sup>; and 2) the phospholipid and fatty acid profiles of membranes from both cardiac and skeletal muscle may be altered (*i.e.*, secondarily).<sup>29</sup>

The ryanodine-sensitive calcium-release channel has been considered to be primarily localized in muscle, but recent studies have indicated that ryanodine binding sites also exist in brain and liver microsomes.<sup>30,31</sup> Thus, it is of interest to determine whether abnormal regulation of  $[\text{Ca}^{2+}]_i$ , induced by halothane, is a common defect in cells other than those from skeletal muscle fibers isolated from swine susceptible to MH. In the current study, we set out to determine whether  $\text{Ca}^{2+}$  mobilization was different in hepatocytes prepared from swine susceptible to MH compared with their normal counterparts. We observed significant enhancement of the effects of halothane on  $\text{Ca}^{2+}$  mobilization within hepatocytes isolated from swine susceptible to MH compared with those from normal animals.

### Materials and Methods

#### ANIMALS

This protocol was approved by the Institutional Animal Care and Use Committee. Swine were obtained from a special breeding program at the University of Minnesota. Normal swine were mongrels, and swine susceptible to MH were purebred Pietrains. The susceptible animals all reacted to halothane (3% for 5 min or less) with stiffness or other signs of MH during a barnyard halothane test. Both the normal animals and those susceptible to MH were used before this in a study in which they were exposed to desflurane (1-2 MAC for 1 h).<sup>32</sup> The animals were housed for a minimum of 9 days between studies, and the housing conditions, diet, and other factors were identical for each animal during this period. The animals were fasted on the morning of use, and the biopsy procedures were performed at the same time of day. The animals were anesthetized with sodium thiopental (20-40 mg/kg), and fresh specimens of skeletal muscle were

\* Assistant Professor of Anesthesiology, Mayo Medical School.

† Research Fellow, Department of Pharmacology, Mayo Clinic.

‡ Research Associate, Department of Pharmacology, Mayo Clinic.

§ Visiting Scientist, Department of Pharmacology, Mayo Clinic.

¶ Professor of Pharmacology, Mayo Medical School.

Received from the Departments of Anesthesiology and Pharmacology, Mayo Clinic, Rochester, Minnesota. Accepted for publication October 31, 1990. Supported by Anaquest (P.A.I. and D.J.W.) and CA42286 (G.P.) from the General Medical Sciences Institute and the National Cancer Institute.

Address reprint requests to Dr. Iaizzo: Department of Anesthesiology, University of Minnesota, 420 Delaware Street, SE, Minneapolis, Minnesota 55455.

obtained for *in vitro* contracture testing. With the use of this test and/or the *in vivo* trigger of an episode of MH by succinylcholine, the animals were confirmed as normal or susceptible to MH.<sup>32</sup> The detailed results of the *in vitro* contracture testing for these animals have been presented elsewhere.<sup>32</sup> Briefly, bundles of muscle fibers from the latissimus dorsi were studied and, for the susceptible animals, a contracture of 200 mg or greater was recorded at caffeine concentrations of 2 mM or less and at 0.5% halothane (a positive test result as defined by the European Malignant Hyperthermia Group).<sup>33</sup> In contrast, muscle bundles from the normal swine did not elicit contractures with amplitudes greater than 200 mg until caffeine concentrations were greater than 3 mM and they did not react to halothane (concentrations > 3%).

#### PREPARATION OF HEPATOCYTES

All chemicals and drugs were from Sigma Chemical Company, St. Louis, Missouri, unless indicated otherwise. With the animals under anesthesia and immediately after removal of the skeletal muscle, the whole liver was removed. Promptly, each lobe of liver was perfused with cold Dulbecco's phosphate-buffered saline (PBS; Gibco Laboratories, Grand Island, NY) containing heparin (6,000 units/l) to prevent blood clotting. Hepatocytes were prepared by a two-step perfusion of one liver lobe from each animal (approximately 150 g of tissue). First, the lobe was perfused for approximately 10 min at 37° C with 2 l of Ca<sup>2+</sup>-free PBS containing 25 mM NaHCO<sub>3</sub>, 12.5 mM 2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) (at pH 7.4), and 0.5 mM ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA). Subsequently, the liver lobe was perfused for 20–25 min at 37° C with PBS containing 25 mM NaHCO<sub>3</sub>, 12.5 mM HEPES (pH 7.4), 4 mM CaCl<sub>2</sub>, and 1 g/L collagenase B (Boehringer Mannheim, Mannheim, Germany). Both perfusion media were gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The collagenase-digested liver tissue was gently minced and resuspended in a Krebs bicarbonate buffer containing 110.0 mM NaCl, 4.0 mM KCl, 1.0 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 24.0 mM NaHCO<sub>3</sub>, and 25.0 mM HEPES. Hepatocytes were separated from tissue debris by filtration through a 250-μm nylon mesh and collected by centrifuging at 800 × g for 2 min at 4° C before being washed once with Krebs bicarbonate buffer. The hepatocytes were then resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco) to a concentration of 6 × 10<sup>6</sup> cells/ml, at which time the approximate viability was estimated to be 80% by trypan blue exclusion. The approximate yield of hepatocytes was 100 × 10<sup>6</sup> cells/150 g tissue.

#### <sup>45</sup>Ca<sup>2+</sup> RELEASE BY PERMEABILIZED HEPATOCYTES

Isolated 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<sub>2</sub>, and 10 mM HEPES/KOH [pH 7.0])<sup>34</sup> and then incubated at 37° C for 20 min in uptake buffer containing 0.005% saponin. The saponin was removed by washing the cells three times in saponin-free uptake buffer. The degree of hepatocyte permeability induced by the saponin treatment, measured by trypan blue penetration, was greater than 90% for each liver preparation studied (n = 6). The cell concentration was determined on a hemocytometer and adjusted with buffer to obtain a final concentration of 5 × 10<sup>6</sup> cells/ml.

An aliquot of hepatocytes was incubated at 37° C with two volumes of uptake buffer containing 5% polyethylene glycol, 0.5 mM 2,4-dinitrophenol, 16 μM antimycin A, 2 μg/ml oligomycin, 1.5 mM adenosine 5' triphosphate (ATP), 3 mM creatine phosphate, 30 μg/ml creatine phosphokinase, and 50 μM CaCl<sub>2</sub>. The free [Ca<sup>2+</sup>] of this solution was buffered to 100 nM by EGTA.<sup>35</sup> Aliquots (100 μl) of cells were removed at various times to determine the rate of <sup>45</sup>Ca<sup>2+</sup> uptake. The cells were collected on a vacuum filter system (VFM 1; Amicon, Danvers, MA) with the use of glass fiber filters (Whatman GF/A, Maidstone, England) and immediately washed four times with uptake buffer containing 1 mM LaCl<sub>3</sub> (which is considered a potent Ca<sup>2+</sup> channel-blocking agent used to minimize any additional release of Ca<sup>2+</sup> from internal stores). The filters were removed and placed in scintillation vials to which 0.5 ml of a digestion solution containing 0.1 N Na[OH], 2% Na<sub>2</sub>CO<sub>3</sub>, and 1% sodium dodecyl sulfate was added. After a 1-h incubation at 55° C, 10 ml of liquid scintillation cocktail (Ultima Gold®, Packard, Downers Grove, IL) was added and radioactivity determined.

When the uptake of <sup>45</sup>Ca<sup>2+</sup> by the hepatocytes at 37° C had reached a maximum (approximately 8 min), the effects of various agents on <sup>45</sup>Ca<sup>2+</sup> release were determined. First, an initial 100-μl sample was removed from the incubation mixture. Fifteen seconds later the releasing agents were added to the incubated hepatocytes, and 45 s later a second sample was collected. The amount of <sup>45</sup>Ca<sup>2+</sup> release was determined as the percentage of <sup>45</sup>Ca<sup>2+</sup> remaining at 9 min, relative to the amount of <sup>45</sup>Ca<sup>2+</sup> within the cells sampled after 8 min. For a given preparation, all release values were corrected for either additional uptake or spontaneous release of <sup>45</sup>Ca<sup>2+</sup> that may have occurred between the 8- and 9-min sample periods. The releasing agents were added as dilutions of uptake buffer and were as follows: 1) halothane (Ayerst Laboratories, New York, NY) as dilutions of a saturated aqueous buffer (~12% or ~2.6 mM halothane); 2) 10 μM 1,4,5-inositol triphosphate (IP<sub>3</sub>); 3) 10 μM guanosine-5'-triphosphate (GTP); and 4) 100 μM cis-5,8,11,14-eicosatetraenoic acid (C<sub>20:4</sub>, arachidonic acid). The final concentration of halothane in the incubation solutions was determined by gas chromatography, and the values for a given dilution were reproducible (±0.2% halothane).<sup>36</sup>

The absolute amount of <sup>45</sup>Ca<sup>2+</sup> uptake by the hepatocytes was determined with the use of the calculation described by Gill and Chuen.<sup>37</sup> Individual experiments were conducted without the examiners' prior knowledge (*i.e.*, in a blinded fashion) of the genetic status of the animal. The radioactivity measurements were obtained 12–14 h after sampling; thus, actual release information was not available at the time of sampling.

#### [Ca<sup>2+</sup>]<sub>i</sub> MEASUREMENT IN INTACT HEPATOCYTES

The aequorin loading technique was a modification of methods previously used to incorporate aequorin into rat hepatocytes.<sup>34</sup> A suspension of fresh hepatocytes, 5 × 10<sup>6</sup> cells/ml from either a normal animal or one susceptible to MH, 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. The final wash buffer also contained 1 mM EGTA. The hepatocytes were then suspended in 0.5 ml of solution containing 5 × 10<sup>-5</sup> M aequorin (purchased from Dr. J. Blinks, Mayo Clinic, Rochester, MN), 0.15 M KCl, and 50 mM HEPES (*pH* 7.4), and incubated with gentle shaking for 10 min at 4° C before centrifugation at 800 × *g* for 10 s. The cells were then suspended in DMEM containing 10% fetal calf serum and plated at 5–6 × 10<sup>6</sup> cells per 35-mm culture dish (Becton Dickinson, Lincoln Park, NJ). The hepatocytes were maintained for 20–24 h at 37° C and gassed with humidified 95% air and 5% CO<sub>2</sub>. Three hours before use, the cells were incubated in DMEM without fetal calf serum.

An estimate of [Ca<sup>2+</sup>]<sub>i</sub> 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 radiofrequency interference-shielded photomultiplier tube (9635QA; Thorn EMI, Fairfield, NJ) in a light-tight chamber. The change in current resulting from the emission of photons was converted to a voltage. All signals were then recorded with 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<sub>2</sub> and the total light signal integrated. Changes in [Ca<sup>2+</sup>]<sub>i</sub> were estimated with the use of the methods reported previously.<sup>38,39</sup> 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 s<sup>-1</sup> at 37° C) to obtain a calculated peak intensity (*L*<sub>max</sub>) 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*<sub>max</sub> was calculated and compared with an aequorin Ca<sup>2+</sup> concentration–light emission curve which was constructed assuming an intracellular free Mg<sup>2+</sup> concentration of 1 mM.<sup>39</sup>

When the resting levels of aequorin luminescence were considered stable, the effects of halothane on [Ca<sup>2+</sup>]<sub>i</sub> were determined. As before, the halothane was added as various dilutions of a saturated aqueous buffer, and the final concentrations of halothane in the incubation solutions were determined by gas chromatography.<sup>36</sup> A minimum of triplicate determinations were made for each preparation of hepatocytes at each concentration of halothane.

#### STATISTICAL ANALYSIS

Statistical significance of the data was determined with the use of either a two-way analysis of variance (*e.g.*, for the halothane dose response data) or a Student's *t* test. With the use of these analyses, a *P* value < 0.05 was considered significant.

#### Results

##### <sup>45</sup>Ca<sup>2+</sup> UPTAKE

The uptake of <sup>45</sup>Ca<sup>2+</sup> by saponin-permeabilized swine hepatocytes was maximal after 7 to 10 min of incubation. A <sup>45</sup>Ca<sup>2+</sup> uptake curve for saponin-treated hepatocytes from a normal swine is shown in figure 1. There was no difference in the apparent uptake of <sup>45</sup>Ca<sup>2+</sup> by the hepatocytes isolated from normal swine, compared with those prepared from swine susceptible to MH. Between the 8- and 9-min time periods, for either type of hepatocyte preparation, there was minimal release and/or additional uptake of the <sup>45</sup>Ca<sup>2+</sup>. Between the two groups, the mean responses for all control experiments (*i.e.*, no releasing agent added) were not significantly different: the values were -1.8 ± 10.6% (*n* = 14; mean ± SD) for the hepatocytes from the normal animals and -1.4 ± 9.3% (*n* = 15) for those from the susceptible animals (indicating a slight <sup>45</sup>Ca<sup>2+</sup> uptake in both cases). Thus, it was reasonable to investigate the effects of the putative releasing agents on the release of the <sup>45</sup>Ca<sup>2+</sup> between the 8- and 9-min time periods.

##### <sup>45</sup>Ca<sup>2+</sup> RELEASE

Halothane induced a reproducible, concentration-dependent release of <sup>45</sup>Ca<sup>2+</sup> from hepatocytes of both normal animals and those susceptible to MH. Figure 2 shows the mean values of <sup>45</sup>Ca<sup>2+</sup> release and regression lines fit to the data. There were three animals in each group. Halothane induced a significantly greater release of <sup>45</sup>Ca<sup>2+</sup> at all concentrations in hepatocytes prepared from the animals susceptible to MH (fig. 2).

The putative second messengers IP<sub>3</sub>, GTP, and arachidonic acid induced significant releases of <sup>45</sup>Ca<sup>2+</sup> in saponin-treated hepatocytes from normal swine and those susceptible to MH (*P* < 0.001) (fig. 3). However, only the <sup>45</sup>Ca<sup>2+</sup> release induced by IP<sub>3</sub> was significantly greater in

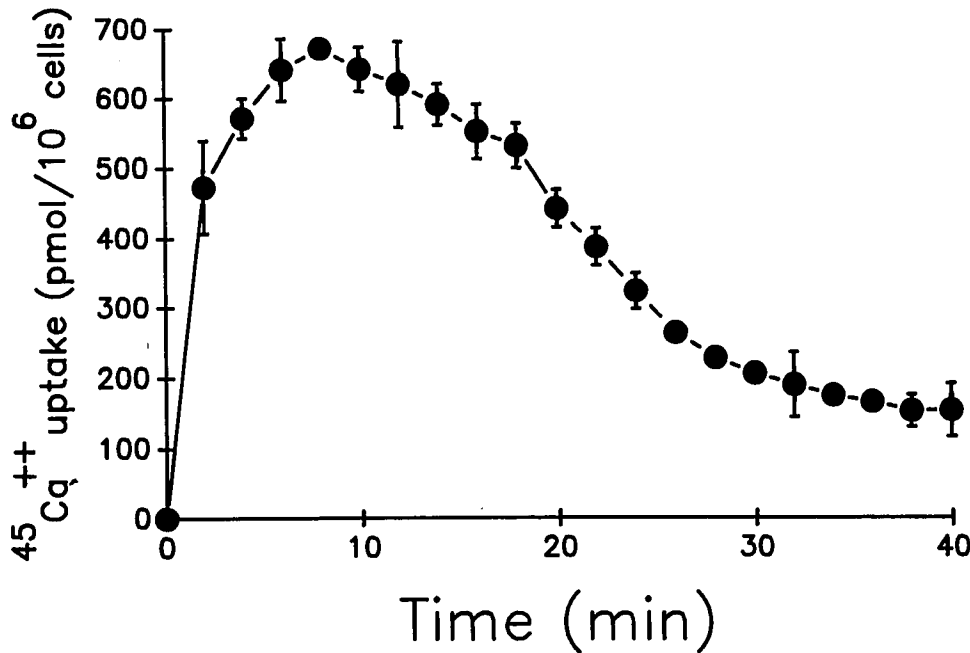


FIG. 1. Uptake of  $^{45}\text{Ca}^{2+}$  by saponin-permeabilized hepatocytes from a normal swine. Values from five samples were averaged at each time point (mean  $\pm$  SD).

the hepatocytes prepared from the animals susceptible to MH ( $P < 0.001$ ):  $23 \pm 6\%$  ( $n = 15$ ) for those from normal swine versus  $33 \pm 9\%$  ( $n = 15$ ) for those prepared from the swine susceptible to MH (mean  $\pm$  SD).

INCREASES IN INTRACELLULAR  $[\text{Ca}^{2+}]_i$  WITHIN INTACT HEPATOCYTES

The estimated resting  $[\text{Ca}^{2+}]_i$  was not significantly different for the hepatocytes prepared from the two groups

of animals, and they were  $0.16 \pm 0.04 \mu\text{M}$  ( $n = 12$ ) for the normal swine and  $0.15 \pm 0.07 \mu\text{M}$  ( $n = 14$ ) for the animals susceptible to MH (mean  $\pm$  SD).

The administration of halothane caused transient increases in  $[\text{Ca}^{2+}]_i$  in hepatocytes prepared from both normal and susceptible animals. The amplitudes and overall areas of the  $\text{Ca}^{2+}$  transients were dose-dependent and were larger for the hepatocytes prepared from an animal susceptible to MH ( $P < 0.01$ ) (fig. 4). The calculated averages of the peak  $[\text{Ca}^{2+}]_i$  amplitudes and normalized areas of

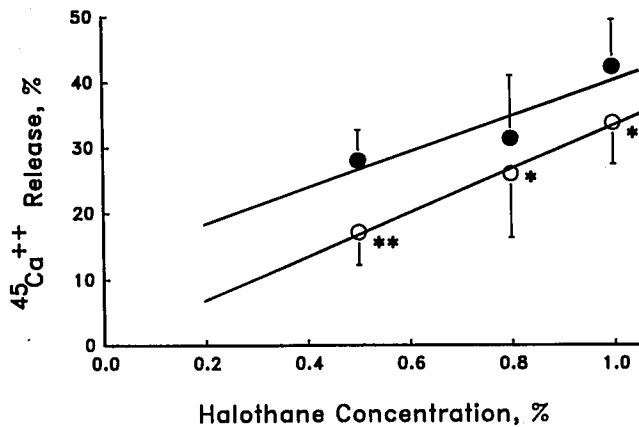


FIG. 2. The effects of halothane on  $^{45}\text{Ca}^{2+}$  release in hepatocytes prepared from normal swine (open circles) and swine susceptible to MH (filled circles). The mean values (bars indicate SD) of anesthetic-induced release ( $n = 15$  for three animals from each group) were plotted against the halothane concentration. At each halothane concentration (*in vivo* equivalents), significantly more  $^{45}\text{Ca}^{2+}$  was released from the hepatocytes prepared from the animals susceptible to MH ( $*P < 0.05$  and  $**P < 0.001$ ). Regression lines were fit to the data: the correlation coefficients were 0.997 for the data from the normal swine and 0.916 for the susceptible group.

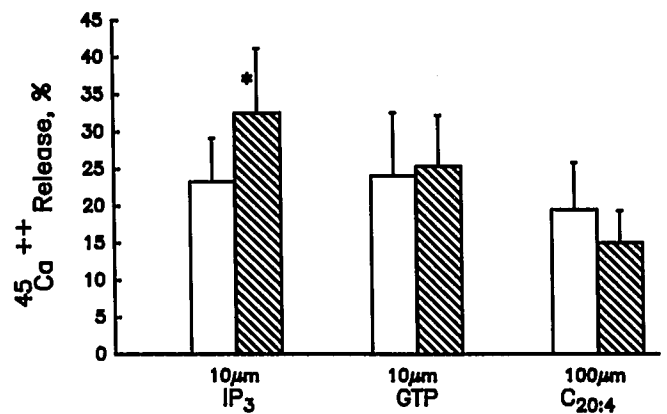
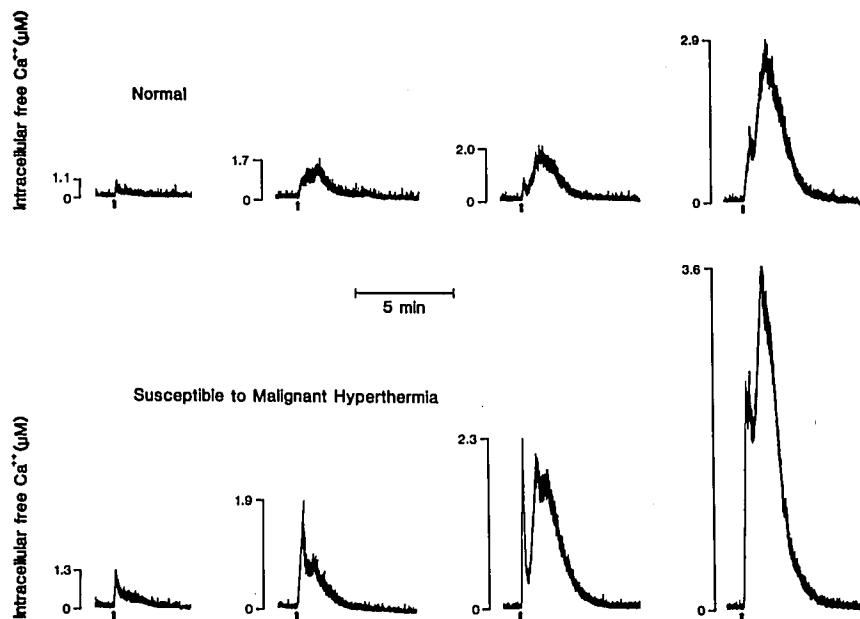


FIG. 3. The release of  $^{45}\text{Ca}^{2+}$  from hepatocytes prepared from normal swine and those susceptible to MH, induced by several agents considered important in intracellular second messenger systems. Mean values (and SD) were derived from data obtained from hepatocytes prepared from three normal animals (open bars) and three animals susceptible to MH (hatched bars). Only the release of  $^{45}\text{Ca}^{2+}$  induced by  $\text{IP}_3$  ( $10 \mu\text{M}$ ) was significantly different ( $*$ ) between the two types of preparations ( $P < 0.001$ ). Arachidonic acid ( $\text{C}_{20:4}$ ,  $100 \mu\text{M}$ ) and GTP ( $10 \mu\text{M}$ ) induced significant ( $P < 0.001$ ) but similar releases of  $^{45}\text{Ca}^{2+}$  in hepatocytes prepared from both types of animals.

FIG. 4. Larger transient increases in [Ca<sup>2+</sup>]<sub>i</sub> induced by halothane in intact hepatocytes prepared from swine susceptible to MH. Swine hepatocytes were loaded with aequorin using a low Ca<sup>2+</sup> centrifugation method and were plated in culture dishes for at least 20 h before study. Each set of records represents data recorded for groups of cells prepared from the same liver. (Top) The response of hepatocytes prepared from a normal animal to increasing concentrations of halothane. (Bottom) Responses recorded from cells prepared from an animal susceptible to MH. The increasing halothane concentrations were approximately 0.5, 1, 2, and 6% (or approximately 0.11, 0.22, 0.44, and 1.32 mM) respectively. The arrows indicate the onset of halothane administration.



the recorded [Ca<sup>2+</sup>]<sub>i</sub> transients after the administration of various doses of halothane are provided in table 1. When all data are averaged together, the peak [Ca<sup>2+</sup>]<sub>i</sub> amplitudes and normalized areas were, respectively, 1.3- and 3.1-fold greater for the responses recorded from the hepatocytes prepared from swine susceptible to MH. Although the durations of the Ca<sup>2+</sup> transients were similar (fig. 4), the larger areas for those recorded from the susceptible animals indicate that the overall amount of Ca<sup>2+</sup> that was released was much greater.

Discussion

We have previously reported that halothane and IP<sub>3</sub> each produced a significant release of <sup>45</sup>Ca<sup>2+</sup> from the nonmitochondrial stores of saponin-permeabilized rat hepatocytes.<sup>34</sup> In addition, we reported that volatile anesthetic agents, in a dose-dependent manner, induced transient increases in [Ca<sup>2+</sup>]<sub>i</sub> in intact rat hepatocytes that were considered nontoxic.<sup>40</sup> Similarly, in the current study, halothane caused the release of <sup>45</sup>Ca<sup>2+</sup> in a dose-dependent manner from saponin-permeabilized swine hepatocytes. However, the Ca<sup>2+</sup> mobilization induced by both halothane and IP<sub>3</sub> was enhanced in hepatocytes prepared from swine susceptible to MH, compared with those from normal swine. Furthermore, the amplitudes of the calcium transients induced by halothane (monitored by the use of aequorin) were larger in hepatocytes prepared from swine susceptible to MH. These data support the hypothesis that the mobilization of intracellular Ca<sup>2+</sup> within hepatocytes is affected in MH.

Most reports on MH in which Ca<sup>2+</sup> regulation has been studied in cells other than skeletal muscle have focused on cells that may be useful in the development of non-invasive tests to determine susceptibility to MH. For example, abnormal Ca<sup>2+</sup> regulation has been reported to occur in red blood cells,<sup>18</sup> platelets,<sup>5</sup> and lymphocytes<sup>7,8,13</sup> isolated from humans or swine susceptible to MH. Unfortunately, these findings, as they relate to our understanding of the pathophysiology of MH, have been deemphasized because identified differences were not reliable in determining susceptibility to MH. Other reports have described abnormal cellular functions, not directly related to the regulation of [Ca<sup>2+</sup>]<sub>i</sub>, in a variety of cell types.<sup>2,3,6,10,12,14,17,19</sup> For example, in a recent study of

TABLE 1. Peak Amplitudes and Normalized Areas of the [Ca<sup>2+</sup>]<sub>i</sub> Transients Detected from Aequorin-loaded Intact Hepatocytes

Halothane (%)	n	Normal	n	Susceptible to Malignant Hyperthermia
Peak amplitude [Ca <sup>2+</sup> ] <sub>i</sub> (μM)				
0.5	3	1.04 ± 0.07	4	1.27 ± 0.17
1	3	1.40 ± 0.10	3	1.87 ± 0.05*
2	3	1.80 ± 0.17	3	2.30 ± 0.20*
6	5	2.58 ± 0.26	3	3.33 ± 0.23*
Normalized area (μV · ms · L <sub>max</sub> <sup>-1</sup> )				
0.5	3	6.4 ± 2.9	4	24.7 ± 17.5
1	3	9.0 ± 7.5	3	88.0 ± 17.4
2	3	105.7 ± 40.1	3	224.2 ± 53.9*
6	5	333.8 ± 92.9	3	641.1 ± 69.3*

The peak amplitudes and areas of the [Ca<sup>2+</sup>]<sub>i</sub> transients were obtained by digital analysis of records of the original strip chart data. The peak [Ca<sup>2+</sup>]<sub>i</sub> were estimated using the methods previously reported.<sup>38,39</sup> The areas of the [Ca<sup>2+</sup>]<sub>i</sub> transients (mV · s) were normalized by dividing by the L<sub>max</sub> determined in each experiment. Values are mean ± SD of n determinations. Halothane concentrations (*in vivo* equivalents) were determined by gas chromatography.

\* Significantly different between the groups at P ≤ 0.01.

liver cells, Duthie *et al.* reported that microsomes from MH-susceptible swine have an enhanced occurrence of free radical-mediated lipid peroxidation.<sup>3</sup> Nevertheless, a generalized alteration in regulation of  $[Ca^{2+}]_i$  and other specific cellular functions in multiple organ systems within animals or humans susceptible to MH is an appealing hypothesis to explain the reported lower tolerance to physical or chemical manipulation of humans or swine susceptible to MH.<sup>4,41</sup>

It has been reported recently that the calcium-release channel (*i.e.*, the ryanodine receptor) located in the sarcoplasmic reticulum of skeletal muscle is defective in both swine and humans susceptible to MH.<sup>11,23-28</sup> The calcium-release channel is considered a tetrameric assembly of subunits with molecular weights of approximately 560,000.<sup>42,43</sup> It is suggested that a genetic defect of this channel is responsible for the abnormal regulation of  $[Ca^{2+}]_i$  that occurs within skeletal muscle in MH.<sup>9,11,23-28</sup> However, the ryanodine receptor is only a candidate gene, and other possibilities have been described.<sup>44</sup>

Formerly, this ryanodine-sensitive channel was considered to exist primarily in the sarcoplasmic reticulum of skeletal, cardiac, and smooth muscle. Recently, it has been reported that ryanodine will bind to sites within brain and liver microsomes, presumably to calcium channels.<sup>30,31</sup> The enhanced mobilization of intracellular  $Ca^{2+}$  induced by halothane in hepatocytes prepared from MH-susceptible swine may result from the presence of the same defective calcium-release channel as in muscle. However, this needs verification and such information should be available soon, now that the complete cDNA sequence of the human skeletal muscle calcium-release channel has been determined.<sup>43</sup> It is possible that the liver and skeletal muscle ryanodine receptors are not the same and perhaps are encoded on different chromosomes.

On the other hand, it has been shown recently that the  $IP_3$  receptor ( $InsP_3R$ ) of endoplasmic reticulum (isolated from brain) has a considerable amino acid homology with the calcium-release channel of sarcoplasmic reticulum.<sup>45</sup> The enhanced  $Ca^{2+}$  mobilization by  $IP_3$  and halothane that we observed for hepatocytes prepared from swine susceptible to MH might be explained if such channels are present and perhaps also function defectively in these hepatocytes. Consistent with the hypothesis that in MH there is an abnormal second messenger system that includes  $IP_3$ , it was recently reported that a deficiency in inositol 1,4,5-trisphosphate phosphatase activity occurs in MH.<sup>46</sup>

The data presented here may indicate that not all second messenger systems that involve modulations of  $[Ca^{2+}]_i$  are altered in MH. For example, it has been reported that, in normal cells, volatile anesthetics, depending on the cell type, can either activate or inhibit guanine nucleotide binding proteins (G-proteins).<sup>34,47,48</sup> The G-proteins are associated with the adrenoceptor, which, in he-

patocytes, can be considered one of the primary control systems associated with  $Ca^{2+}$  mobilization.<sup>47</sup>  $IP_3$  has been identified as the component of this system directly responsible for the release of  $Ca^{2+}$  from the endoplasmic reticulum.<sup>49,50</sup> The data presented here indicated that  $IP_3$  was capable of stimulating the release of  $^{45}Ca^{2+}$  from nonmitochondrial stores of permeabilized swine hepatocytes but that its ability to do so was enhanced in hepatocytes isolated from swine susceptible to MH. In contrast, GTP and arachidonic acid induced similar releases of  $^{45}Ca^{2+}$  in the hepatocytes prepared from the two groups of swine.

Fluctuations in the concentrations of intracellular GTP were originally postulated to modulate  $[Ca^{2+}]_i$  by a direct effect on the  $IP_3$ -sensitive  $Ca^{2+}$  channels ( $InsP_3R$ ) located on the endoplasmic reticulum, but more recent findings suggest that the effect of GTP is considerably more complex.<sup>49,50</sup> Thus, the action of GTP is not exclusively linked to the same pathway that includes the release of  $Ca^{2+}$  through G-proteins and  $IP_3$ . Likewise, arachidonic acid is thought to induce the release of  $Ca^{2+}$  from endoplasmic reticulum through an alternate pathway. Increases in  $[Ca^{2+}]_i$  are associated with the formation of arachidonic acid, which can be activated by receptor binding of bradykinin.<sup>51,52</sup> On the other hand, the fact that GTP and arachidonic acid induced similar releases of  $^{45}Ca^{2+}$  in hepatocytes prepared from both groups of animals and that  $IP_3$  induced a greater release in the animals susceptible to MH may be used as additional evidence that GTP's effect on endoplasmic reticulum is somehow separate from that of  $IP_3$ .<sup>3</sup>

Nevertheless, the exact mechanism by which halothane causes the release of  $Ca^{2+}$  within swine hepatocytes is not known and several possibilities need be considered. For example, halothane may have a direct effect on the endoplasmic reticulum because a similar response has been reported for isolated vesicles of swine sarcoplasmic reticulum.<sup>22</sup> However, halothane may also modify the function of the surface membrane and associated second messenger systems. It has been reported that various properties of the surface membrane are altered in MH.<sup>6,11,17,19</sup>

It is important to consider how information obtained from the animal model for MH, the swine, relates to the human form of this disorder. Although most would agree that the swine is the best animal model for MH, there is question as to whether or not the genetic defect related to the porcine MH syndrome is somehow more complex. Gronert summarized that MH inheritance in swine may be best described as multifactorial,<sup>53</sup> whereas in humans the genetic defect is considered to be isolated within the DNA sequence for the calcium-release channel.<sup>23,25-27</sup> In contrast, we recently described that when the German Landrace porcine model of MH was studied by *in vitro* contracture testing and methods from the field of food science, the mode of inheritance was considered the same

as in humans, autosomal dominant.<sup>54</sup> Regardless of the mode, this then raises the question as to what type of animal would be an appropriate control for experiments such as the one described here. A littermate of a susceptible animal that was classified as normal by an *in vivo* halothane challenge and/or by *in vitro* contracture testing could, in fact, have a partially defective genome associated with, but not primary to, MH. This type of animal may be an inappropriate control. On the other hand, if the control group is chosen as a pure-bred strain of swine (e.g., Yorkshire), the genotype of certain traits unrelated to MH but associated with cellular processes being investigated (i.e., Ca<sup>2+</sup> mobilization in hepatocytes) may be significantly different from that of the MH group, thus also rendering this group of animals as questionable for use as controls. Nevertheless, when trying to determine the relevance of information obtained from swine studies to humans or *visa versa*, one must remember that humans may best be described as mongrels. One might contend that mongrel swine may be the best control population because the study population should contain a wide spectrum of variability (including mutations related to MH but not related to *in vivo* or *in vitro* detection). In the current study, mongrel animals were used; hence, one might argue either for or against this being an inherent limitation of this study.

In conclusion, the studies described here were interpreted to indicate that an abnormal Ca<sup>2+</sup> homeostasis exists in hepatocytes isolated from swine susceptible to MH, which supports the hypothesis that membrane systems from multiple organs are affected in this genetic disorder. In addition, Ca<sup>2+</sup> release from the endoplasmic reticulum of hepatocytes may be induced through more than one pathway and/or population of channels, not all of which might be altered in MH. Additional studies will be required to identify the mechanism(s) governing the abnormal regulation of [Ca<sup>2+</sup>]<sub>i</sub> thought to exist in hepatocytes from MH-susceptible swine.

The authors thank Dr. R. Palahniuk for his comments.

### References

1. Gronert GA, Mott J, Lee J: Aetiology of malignant hyperthermia. *Br J Anaesth* 60:253-267, 1988
2. Cheah KS, Cheah AM, Fletcher JE, Rosenberg H: Skeletal muscle mitochondrial respiration of malignant hyperthermia-susceptible patients. Ca<sup>2+</sup>-induced uncoupling and free fatty acids. *Int J Biochem* 21:913-920, 1989
3. Duthie GG, McPhail DB, Arthur JR, Goodman BA, Morrice PC: Spin trapping of free radicals and lipid peroxidation in microsomal preparations from malignant hyperthermia susceptible pigs. *Free Radic Res Commun* 8:93-99, 1990
4. Ervasti JM, Classens MT, Mickelson JR, Louis CF: Altered transverse tubule dihydropyridine receptor binding in malignant hyperthermia. *J Biol Chem* 264:2711-2717, 1989
5. Hofmann JG, Buttner C, Till U: Sensitivity of the adenine nucleotide metabolism of platelets from malignant hyperthermia patients to halothane. *Biomed Biochim Acta* 4:343-350, 1989
6. Iazzo PA, Lehmann-Horn F, Taylor SR, Gallant EM: Malignant hyperthermia: Effects of halothane on the surface membrane. *Muscle Nerve* 12:173-179, 1989
7. Klip A, Britt BA, Elliott ME, Pegg W, Frodis W, Scott E: Anaesthetic-induced increase in ionized calcium in blood mononuclear cells from malignant hyperthermia patients. *Lancet* 8531:463-466, 1987
8. Klip A, Mills GB, Britt BA, Elliott ME: Halothane-dependent release of intracellular Ca<sup>2+</sup> in blood cells in malignant hyperthermia. *Am J Physiol* 258:C495-C503, 1990
9. Mickelson JR, Gallant EM, Litterer LA, Johnson KM, Rempel WE, Louis CF: Abnormal sarcoplasmic reticulum ryanodine receptor in malignant hyperthermia. *J Biol Chem* 263:9310-9315, 1988
10. Mickelson JR, Thatte HS, Beaudry TM, Gallant EM, Louis CF: Increased skeletal muscle acetylcholinesterase activity in porcine malignant hyperthermia. *Muscle Nerve* 10:723-727, 1987
11. Mickelson JR, Ross JA, Hyslop RJ, Gallant EM, Louis CF: Skeletal muscle sarcolemma in malignant hyperthermia: Evidence for a defect in calcium regulation. *Biochim Biophys Acta* 897:364-376, 1987
12. Niebroj-Dobosz I, Kwiatkowska H, Mayzner-Zawadzka E: Experimental porcine malignant hyperthermia: Macromolecular characterization of muscle plasma membrane. *Med Biol* 62:250-254, 1984
13. O'Brien PJ, Kalow BI, Brown BD, Lumsden JH, Jacobs RM: Porcine malignant hyperthermia susceptibility: Halothane-induced increase in cytoplasmic free calcium in lymphocytes. *Am J Vet Res* 50:131-135, 1989
14. Ohnishi ST, Katagi H, Ohnishi T, Brownell AKW: Detection of malignant hyperthermia susceptibility using spin label technique on red blood cells. *Br J Anaesth* 61:565-568, 1988
15. Ohnishi ST, Ohnishi T: Halothane induced disorder of red cell membranes of subjects susceptible to malignant hyperthermia. *Cell Biochem Funct* 6:257-261, 1988
16. Patterson DSP, Allen WM: Biochemical aspects of some pig muscle disorders. *Br Vet J* 128:101-111, 1972
17. Ruppertsberg JP, Rudel R: Differential effects of halothane on adult and juvenile sodium channels in human muscle. *Pflugers Arch* 412:17-21, 1988
18. Thatte HS, Mickelson JR, Addis PB, Louis CF: Erythrocyte membrane ATPase and calcium pumping activities in porcine malignant hyperthermia. *Biochem Med Metab Biol* 38:355-365, 1987
19. Weiland SJ, Fletcher JE, Rosenberg H, Qi-Hua G: Malignant hyperthermia: Slow sodium current in cultured human muscle cells. *Am J Physiol* 257:C759-C765, 1989
20. Endo M, Yagi S, Ishizuka T, Horiuti K, Koga Y, Amaha K: Changes in the Ca-induced Ca release mechanism in the sarcoplasmic reticulum of the muscle from a patient with malignant hyperthermia. *Biomed Res* 4:83-89, 1983
21. Iazzo PA, Klein W, Lehmann-Horn F: Fura-2 detected myoplasmic calcium and its correlation with contracture force in skeletal muscle from normal and malignant hyperthermia susceptible pigs. *Pflugers Arch* 411:648-653, 1988
22. Ohnishi ST, Taylor SR, Gronert G: Calcium-induced Ca<sup>++</sup> release from sarcoplasmic reticulum from malignant hyperthermia and normal pig muscle. *FEBS Lett* 161:103-107, 1983
23. Bender K, Senff H, Wicker TF, Spiess-Kieffer C, Lehmann-Horn F: A linkage study of malignant hyperthermia (MH). *Clin Genet* 37:221-225, 1990
24. Fill M, Coronado R, Mickelson JR, Vilven J, Ma J, Jacobson BA, Louis CF: Abnormal ryanodine receptor channels in malignant hyperthermia. *Biophys J* 50:471-475, 1990

25. Knudson CM, Mickelson JR, Louis CF, Campbell KP: Distinct immunopeptide maps of the sarcoplasmic reticulum  $\text{Ca}^{2+}$  release channel in malignant hyperthermia. *J Biol Chem* 265:2421-2424, 1990
26. MacLennan DH, Duff C, Zorzato F, Fujii J, Phillips M, Korneluk RG, Frodis W, Britt BA, Worton RG: Ryanodine receptor gene is a candidate for predisposition to malignant hyperthermia. *Nature* 344:559-561, 1990
27. McCarthy TV, Healy JMS, Lehane M, Heffron JJA, Deufel R, Lehmann-Horn F, Farrall M, Johnson K: Localization of the malignant hyperthermia susceptibility locus to human chromosome 19q12-13.2. *Nature* 343:562-563, 1990
28. Mickelson JR, Litterer LA, Jacobson BA, Louis CF: Stimulation and inhibition of [ $^3\text{H}$ ]ryanodine binding to sarcoplasmic reticulum from malignant hyperthermia susceptible pigs. *Arch Biochem Biophys* 278:251-257, 1990
29. Seewald MJ, Eichinger HM, Iaizzo PA: Malignant hyperthermia: An altered phospholipid and fatty acid composition in muscle membrane. *Acta Anaesthesiol Scand* (in press)
30. Ashley RH: Activation and conductance properties of ryanodine-sensitive calcium channels from brain microsomal membranes incorporated into planar lipid bilayers. *J Membr Biol* 111:179-189, 1989
31. Shoshan-Barmatz V: High affinity ryanodine binding sites in rat liver endoplasmic reticulum. *FEBS Lett* 263:317-320, 1990
32. Wedel DJ, Iaizzo PA, Milde JH: Desflurane is a trigger of malignant hyperthermia in susceptible swine. *ANESTHESIOLOGY* 74:508-512, 1991
33. European Malignant Hyperpyrexia Group: A protocol for the investigation of malignant hyperpyrexia (MH) susceptibility. *Br J Anaesth* 56:1267-1269, 1984
34. Iaizzo PA, Seewald M, Powis G, Van Dyke RA: The effects of volatile anesthetics on  $\text{Ca}^{++}$  mobilization in rat hepatocytes. *ANESTHESIOLOGY* 72:504-509, 1990
35. Fabiato A: Computer programs for calculating total from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Methods Enzymol* 31:378-417, 1988
36. Van Dyke RA, Wood CL: Binding of radioactivity from  $^{45}\text{C}$ -labeled halothane in isolated perfused rat livers. *ANESTHESIOLOGY* 38:328-332, 1973
37. Gill DL, Chuen S-H: An intracellular (ATP +  $\text{Mg}^{++}$ )-dependent calcium pump within the N1E-115 neuronal cell line. *J Biol Chem* 260:9289-9297, 1985
38. Allen DG, Blinks JR: The interpretation of light signals from aequorin-injected skeletal and cardiac muscle cells: A new method of calibration, Detection and Measurement of Free  $\text{Ca}^{2+}$  in Cells. Edited by Ashley CC, Campbell AK. Amsterdam, Elsevier-North Holland, 1979, pp 159-174
39. Alvarez-Leefmans FG, Giraldez F, Gamino SM: Intracellular free magnesium in excitable cells: Its measurement and its biologic significance. *Can J Physiol Pharmacol* 65:915-925, 1987
40. Iaizzo PA, Olsen RA, Seewald MJ, Powis G, Stier A, Van Dyke RA: Transient increases of intracellular  $\text{Ca}^{2+}$  induced by volatile anesthetics in rat hepatocytes. *Cell Calcium* 11:515-524, 1990
41. Ørding H, Hald A, Sjøntoft E: Malignant hyperthermia triggered by heating in anaesthetized pigs. *Acta Anaesthesiol Scand* 29:698-701, 1985
42. Lai FA, Misra M, Xu L, Smith HA, Meissner G: The ryanodine receptor- $\text{Ca}^{2+}$  release channel complex of skeletal muscle sarcoplasmic reticulum. *J Biol Chem* 264:16776-16785, 1989
43. Zorzato F, Fujii J, Otsu K, Phillips M, Green NM, Lai Fa, Meissner G, MacLennan DH: Molecular cloning of cDNA encoding human and rabbit forms of the  $\text{Ca}^{2+}$  release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. *J Biol Chem* 265:2244-2256, 1990
44. Levitt RC, McKusick VA, Fletcher JE, Rosenberg H: Gene candidate. *Nature* 345:297-298, 1990
45. Gill DL: Receptor kinships revealed. *Nature* 342:16-18, 1989
46. Foster PS, Gesini E, Claudianos C, Hopkinson KC, Denborough MA: Inositol 1,4,5-trisphosphate phosphatase deficiency and malignant hyperthermia in swine. *Lancet* 8655:124-127, 1989
47. Graham RM, Sena LM, Longabaugh JP, Sawutz DG, Schwartz KR, Homcy CJ: Coupling of the 415-adrenergic receptor to a guanine nucleotide-binding regulation protein by a discrete domain distinct from its ligand recognition site. *Biochim Biophys Acta* 968:119-126, 1988
48. Bernstein KJ, Verosky M, Triner L: Effect of halothane on rat liver adenylate cyclase: Role of cytosol components. *Anesth Analg* 64:531-537, 1985
49. Dawson AP, Comerford JG: Effects of GTP on  $\text{Ca}^{2+}$  movements across endoplasmic reticulum membranes. *Cell Calcium* 10:343-350, 1989
50. Gill DL, Ghoch TK, Mullaney JM: Calcium signalling mechanisms in endoplasmic reticulum activated by inositol 1,4,5-trisphosphate and GTP. *Cell Calcium* 10:363-374, 1989
51. McPhail LC, Clayton CC, Synderman R: A potential second messenger role for unsaturated fatty acids: Activation of  $\text{Ca}^{2+}$ -dependent protein kinase. *Science* 224:622-625, 1984
52. Powis G, Seewald MJ, Sehgal I, Iaizzo PA, Olsen RA: Platelet derived growth factor stimulates non-mitochondrial  $\text{Ca}^{2+}$  uptake and inhibits mitogen-induced  $\text{Ca}^{2+}$  signalling in Swiss 3T3 fibroblasts. *J Biol Chem* 265:10266-10273, 1990
53. Gronert GA: Malignant hyperthermia, Myology. Edited by Engel AG, Banker BQ. New York, McGraw-Hill, 1986, pp 1763-1784
54. Seewald MJ, Eichinger HM, Lehmann-Horn F, Iaizzo PA: Characterization of swine susceptible to malignant hyperthermia by *in vivo*, *in vitro* and *post-mortem* techniques. *Acta Anaesthesiol Scand* (in press)