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ORIGINAL RESEARCH

Effects of isoflurane on measurements of delayed lumininescence in *Acetabularia acetabulum*

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ABSTRACT: The volatile halogenated methyl ethyl ether, isoflurane, used as an anaesthetic, inhibits actin-based dynamics directly or indirectly in animal cells. In plant cells, most intracellular movements are related to actin pathways. We have used isoflurane in a unicellular alga, *Acetabularia acetabulum*, to test the dynamics of choloroplast organization. By measuring the delayed luminescence, we found that isoflurane worked efficiently in the unicellular organism and showed dose- and time-course-dependent actin-inhibition patterns. When *A. acetabulum* was treated with saturated solutions of isoflurane in artificial seawater (defined as 100% isoflurane) for 3 or 6 min, the delayed luminescence (DL) was decreased and was never recovered. In contrast, if treated with 75% diluted isoflurane, the DL was firstly inhibited and then recovered several hours later, and if treated with 50% diluted isoflurane, the change of DL was small. Our work proved that isoflurane can affect actin-related pathways in both animals and plants. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: isoflurane; *Acetabularia acetabulum*; delayed luminescence

INTRODUCTION

All organisms emit weak light spontaneously at very low rates, which is called luminescence. When illuminated with a pulse of light, a much higher rate of re-emission occurs and decays hyperbolically within seconds to minutes. This phenomenon is called delayed luminescence (DL) and its lifetime extends from 10^{-7} s to more than 10 s. In the past, it has been found that there was a close connection between the state of living organisms and their DL in many biological systems (1,2); by measuring DL, information can be obtained about energy-transfer pathways (3).

The giant unicellular green alga, *Acetabularia*, has been used as a model to examine dynamic rearrangements of chloroplasts, and energy-transfer pathway. Chloroplasts of *Acetabularia* are tied together by flexible as well as contractile links, and move along in more or less orderly rows, probably as a consequence of their interconnection by long tubular membrane bridges (4). The interconnections of chloroplasts are important for the functional excitation exchange kinetics of DL, particularly at long periods after illumination (5).

Isoflurane is an anaesthetic that inhibits actin-based dynamics directly or indirectly in animal cells (6). In plant cells, the majority of intracellular movement is based on actin (5), and so we used isoflurane to perturb the actin-based chloroplast movement. By measuring the DL of chloroplasts, we found that isoflurane worked efficiently in the alga and displayed a dosage-dependent pattern.

MATERIALS AND METHODS

Acetabularia acetabulum (L.) cultures were kindly provided by Professor F. A. Popp and R. Van Wijk of the International Institute of Biophysics. They were maintained in artificial sea water, Provasoli's (ASP12) marine medium (Sigma), with a dark–light cycle of 12 h–12 h, at a light intensity of 8 W/m^2 provided by Nathura Tropiclite lamps, in an incubator maintained at a temperature of 20 ± 2°C. The *Acetabularia* cells used for experiments were 2–3 cm long and had not yet formed a cap. An isoflurane-saturated solution in artificial seawater was defined to be 100% isoflurane, and was used to prepare the following: 75% isoflurane (100% isoflurane:fresh artificial seawater = 3:1), 50% isoflurane (100% isoflurane:fresh artificial seawater = 1:1).

The experimental set-up for measuring DL from biological systems has been described in a previous paper (5). Photon counts were stored in a 4096-channel scaler with a minimum dwell time of 2 μs; in our operational mode, each channel of the scaler records the number of pulses counted in a 200 ms time interval. The data presented here were obtained from the values accumulated

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in the first 100 acquisition channels, corresponding to 20 s. The radiation emitted from the quartz cuvette, filled with artificial seawater, was taken as background and was subtracted from the test measurements.

Acetabularia cells were incubated in standard culture medium before measuring DL. In each experiment, only a single individual *Acetabularia* cell was placed in fresh artificial seawater in a quartz cuvette of about 1.5 mL and put into the light-tight chamber positioned in front of the PMT, where the measurements of DL were performed. This was replaced using isoflurane solution instead of fresh culture medium for treatment periods of 3, 6 or 9 min. The cell was then washed and put into fresh culture medium and further measurements of DL performed, immediately and several minutes, hours or days later. Measurements were also made immediately after reincubation in standard culture medium following treatment with isoflurane. All experimental points represent the means of three replicate experiments. Photoexcitation was by illumination with white light from a tungsten (150 W) lamp. To obtain different relative light intensities, neutral grey Schott filters were used: A, $\tau = 0.50$, D = 0.3; B, $\tau = 0.25$, D = 0.6; C, $\tau = 0.10$, D = 1.0; D, $\tau = 0.01$, D = 2.0; E, $\tau = 0.005$, D = 2.3; F, $\tau = 0.0025$, D = 2.6.

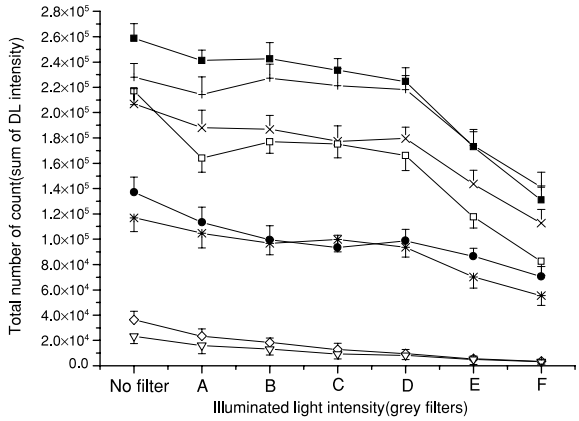

Figure 1. DL from *Acetabularia* cells after excitation by different intensity white light and under different isoflurane treatment conditions: (■), standard condition; (+) immediately after reincubation in standard culture medium after addition of 50% isoflurane and incubation for 3 min; (×) 50% isoflurane, 9 min; (□), 75% isoflurane, 3 min; (●), 75% isoflurane, 6 min; (*), 75% isoflurane, 9 min; (◇), 100% isoflurane, 3 min; (▽), 100% isoflurane, 6 min. Grey Schott filters: A, $\tau = 0.50$, D = 0.3; B, $\tau = 0.25$, D = 0.6; C, $\tau = 0.10$, D = 1.0; D, $\tau = 0.01$, D = 2.0; E, $\tau = 0.005$, D = 2.3; F, $\tau = 0.0025$, D = 2.6. Values represent mean ± SD from three experiments.

RESULTS AND DISCUSSION

The giant unicellular green alga *Acetabularia* has been recognized for many years as an exceptionally favourable organism for examining the dynamic rearrangements of chloroplasts (5). It is known that chloroplast movement is connected with the underling cytoskeleton. In the plant kingdom, the majority of intracellular movements are based on actin. The actin cytoskeleton has been identified as a key downstream effector of external or internal stimuli in a wide range of biological phenomena in plants (7). The cytoskeleton of *Acetabularia* consists of a system of parallel, axially orientated, cortical actin bundles (5). Chloroplasts and mitochondria in normal vegetative *Acetabularia* cells are in a parallel arrangement and their dynamic organization is characterized by a highly complex streaming (8). According to Jursinic's considerations, the similarity of the DL and fluorescence emission spectra indicates that both fluorescence and DL are from the excited singlet state of chloroplasts (9).

Fig. 1 shows the total number of counts (sum of DL intensity) with white excitation sources of different intensity (Schott filters) of the green unicellular alga *Acetabularia acetabulum* treated with different concentrations of isoflurane. By measuring the delayed luminescence, we found that isoflurane worked efficiently in the unicellular organism and showed dose- and time-course-dependent patterns of DL. First, the total counts of DL are reduced with a decrease in the intensity of the excitation light (with Schott filters), i.e. compared with no filter, DL was decreased to 93 ± 3.4%, 93 ± 5.7%, 90 ± 3.8%, 87 ± 5.4%, 51 ± 8.4%, respectively, with the A, B, C, D, E and F Schott filters. Second, when an *Acetabularia* cell was illuminated with excitation light (no filter) and was treated with 50% isoflurane for 3 or 9 min, DL were decreased to 88 ± 4.2%, 80 ± 4.7% compared with control; when treated with in 75% isoflurane for 3, 6 or 9 min, DL was decreased to 84 ± 4.2%, 53 ± 4.6% and 45 ± 4.2% respectively; when treated with 100% isoflurane for 3 or 6 min, DL was decreased to 14 ± 2.6% or 9 ± 2.2% respectively. When the *Acetabularia* cell was illuminated with an excitation light (Schott filter A) and treated with 50% isoflurane for 3 or 9 min, DL was decreased to 89 ± 5.8% and 78 ± 5.8%, respectively; when treated with 75% isoflurane for 3, 6 or 9 min, DL were decreased to 68 ± 4.6%, 47 ± 5.0% and 43 ± 4.8%, respectively; when treated with 100% isoflurane for 3 or 6 min, DL was decreased to 9.7 ± 2.4% and 6.7 ± 2.7%, respectively. With the B, C, D, E and F Schott films, respectively, the results showed similar trends: the degree of DL suppression increased with increasing concentration of isoflurane and the increase in the incubation time.

Volatile anaesthetics have been shown to affect a variety of ion channels and neurotransmitter receptor subtypes, leading to the suggestion that they produce anaesthesia by acting at many sites simultaneously (6). Ca^{2+} has long been proposed to be a molecule that can control actin dynamics during cellular processes such as

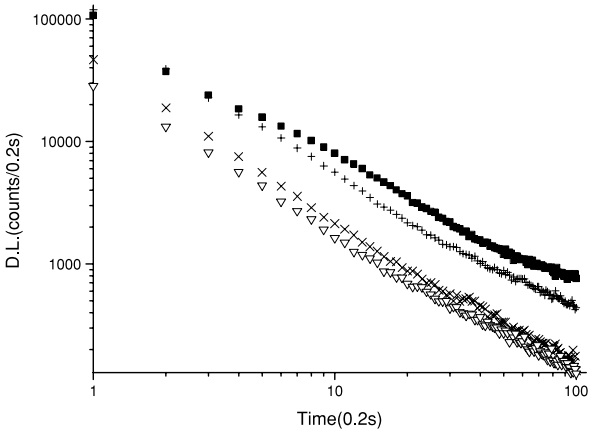

Figure 2. DL decay for an *Acetabularia* cell after 75% isoflurane treatment. Measurements were performed within 3 min of addition of isoflurane; afterwards the cells were washed and put into fresh culture medium, and DL measured immediately (0 min) (+), 3 min later (×) and 6 min later (▽); (■), control (no 75% isoflurane). Curves represent the average of three different *Acetabularia* cells.

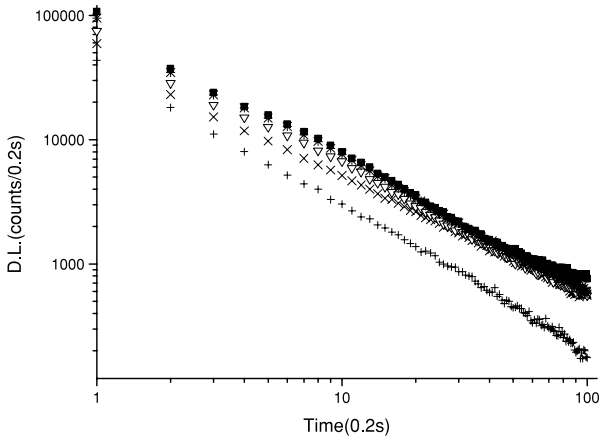

Figure 3. Decrease and recovery of DL for an *Acetabularia* cell after removing 75% isoflurane (9 min treatment). Isoflurane treatment was 9 min, then the cells were washed and put into fresh culture medium, and DL measured 3 min later (+), 2.5 h later (×) and 4 h later (▽), 5 h later (*); (■), control (before adding 75% isoflurane). Curves represent the average of trends of three different *Acetabularia* cells.

polar growth and cytoplasmic streaming (7). Calcium ion influx has indeed been shown to cause rearrangement of the underlying actin cytoskeleton in *Acetabularia* (6). Takagi *et al.* surmised that actin filaments may play important roles in the relocation and anchoring of chloroplasts, and they found that actin filaments could bind to chloroplasts *in vitro* (7). Our data suggest that isoflurane opened Ca^{2+} channels; and then Ca^{2+} inhibited actin-based chloroplasts dynamics in *Acetabularia* cells and stimulated actin-based chloroplasts to associate.

After 3 min of exposure to 75% isoflurane, the cells were reincubated in fresh artificial sea water without isoflurane. We found that, after a 3 min treatment with 75% isoflurane, the DL measured immediately (0 min), and 3 and 6 min post-treatment respectively, was reduced to 85 ± 4.1%, 35 ± 5.0% and 25 ± 4.6% of its initial value (Fig. 2). We also observed that the intensity of DL was first inhibited (Fig. 2) and then recovered 5 h later (Fig. 3). In contrast, after 3 min of exposure to a 100% isoflurane concentration, followed by reincubation of the cells in fresh artificial sea water without isoflurane, a strong and fast decrease of DL total intensity was evident after the subsequent addition of 100% isoflurane (3 min exposure). The total DL intensity decreased sharply to about 20 ± 6.1% of the initial value, but was not stable in this condition. In fact, in the second measurement, performed 7 days after the initial measurement, the total intensity was about 11.13 ± 4.8% of the initial value. If this experimental condition was maintained for 6 min, 9 min or longer periods, the intensity of the DL signal was inhibited further (data not shown), the cells became white and died. DL were decreased and never recovered (Fig. 4). Our data suggest that the 100% isoflurane treatment procedure is an effective means

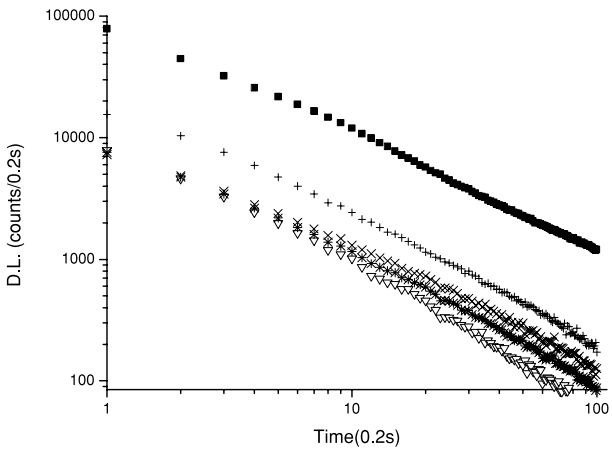

Figure 4. DL decay for an *Acetabularia* cell after isoflurane treatment (100% isoflurane, 3 min). After treatment with isoflurane (3 min), the cells were washed and put into fresh culture medium, and DL measured 3 min later (+), 7 days later (×), 9 days later (▽) and 12 days later (*); (■), control (before adding 100% isoflurane). Curves represent the average of trends of three different *Acetabularia* cells.

for irreversibly disturbing the integrity of the dynamic intracellular network of chloroplasts, resulting in a subsequent loss of cell viability. The capacity of cells to recover was dependent on the concentration of isoflurane and the duration of the treatment. Irreversible damage occurred following treatment with 100% isoflurane and the alga became white, showing a total loss of viability. In contrast, cells reincubated after 75% isoflurane exposure showed complete recovery: their DL was checked for a period of 5 h after the treatment, and no significant change was observed compared with control.

In the search of potential targets for the cellular and molecular actions of volatile anaesthetics, a number of ion channels have been investigated (10). Volatile anaesthetics have been shown to directly and/or indirectly activate (via increases in intracellular Ca^{2+} concentrations) several second messenger systems, including protein kinases C and A and calmodulin (11), and to increase intracellular calcium concentration ($[Ca^{2+}]$) due to a release of calcium from internal stores in hippocampal (12, 13) and cerebrocortical neurons (13), which may alter signalling pathways that influence neurotransmission. In the plant cell, we hypothesized that isoflurane decreased DL by opening a Ca^{2+} channel, and then the Ca^{2+} inhibited actin-based chloroplasts dynamics either directly or indirectly in *Acetabularia* cells, and stimulated actin-based chloroplasts to associate. With the aim of explaining such a connection, new measurements are being planned, using both specific inhibitors of actin filaments and Ca^{2+} channels and emission spectrum of DL. Thus, DL could be used as an experimental technique to investigate anaesthetics that inhibit actin-based dynamics directly or indirectly in plant cells.

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