ELSEVIER

Brain Research Bulletin 69 (2006) 147–152

BRAIN RESEARCH BULLETIN

www.elsevier.com/locate/brainresbull

The effect of sevoflurane on intracellular calcium concentration from cholinergic cells

Ana Cristina Nascimento Pinheiro [a], Renato Santiago Gomez [b,*], Cristina Guatimosim [c], Janice Henriques Silva [a], Marco Antonio Máximo Prado [a], Marcus Vinicius Gomez [a]

[a] *Departamento de Farmacologia, ICB, UFMG, Belo Horizonte, Brazil*

[b] *Departamento de Cirurgia, Sala 4000, Avenida Alfredo Balena 190, Faculdade de Medicina, Universidade Federal de Minas Gerais (UFMG), 30130-100-Belo Horizonte, Minas Gerais, Brazil*

[c] *Departamento de Morfologia, ICB, UFMG, Belo Horizonte, Brazil*

Received 20 August 2005; received in revised form 10 November 2005; accepted 23 November 2005
Available online 19 December 2005

Abstract

The mechanism of action of volatile anesthetics is not completely understood. Calcium release from internal stores may alter signaling pathways that influence neurotransmission. Abnormalities of the regulation of intracellular calcium concentration ($[Ca^{2+}]_i$) from patients with malignant hyperthermia is a hallmark of this syndrome indicating the potential of these agents to interact with proteins involved in Ca^{2+} signaling. In the present study, a cholinergic cell line (SN56) was used to examine whether the release of calcium from intracellular stores occurs in the presence of sevoflurane. Changes in $[Ca^{2+}]_i$ were measured using fluo-4, a fluorescent calcium sensitive dye and laser scanning confocal microscopy. Sevoflurane induced an increase on $[Ca^{2+}]_i$ from SN56 cells. The sevoflurane-induced increase on $[Ca^{2+}]_i$ remained even when the cells were perfused with medium lacking extracellular calcium. However, this effect was abolished by BAPTA-AM, a chelator of intracellular calcium, suggesting the involvement of intracellular Ca^{2+} stores. Using cyclopiazonic acid, an inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, we investigated whether the depletion of intracellular Ca^{2+} stores interfered with the effect of sevoflurane. In the presence of this agent, sevoflurane caused a small but not significant rise on $[Ca^{2+}]_i$ of the SN56 cells. Dantrolene, an inhibitor of ryanodine-sensitive calcium stores did not modify the sevoflurane increase on $[Ca^{2+}]_i$. Carbachol, a drug that releases Ca^{2+} from the IP_3 pool, abolished the effect of sevoflurane. In addition, xestospongin D, a cell-permeant IP_3 receptor antagonist, decreased significantly the sevoflurane increase on $[Ca^{2+}]_i$. Our data suggest that the sevoflurane-induced increase on $[Ca^{2+}]_i$ from SN56 cells occurs through the release of calcium from IP_3-sensitive calcium stores.

Keywords: SN56 cells; Intracellular calcium; Fluo-4; IP_3; Ryanodine; Sevoflurane

1. Introduction

There have been extensive efforts to characterize the mechanism of action of volatile anesthetics, but their molecular and cellular actions remain unclear [8]. Calcium release from internal stores may alter signaling pathways that influence neurotransmission and abnormalities of the regulation of intracellular calcium concentration ($[Ca^{2+}]_i$) is a characteristic found in the skeletal muscle from patients with malignant hyperthermia [19].

It has been documented that volatile anesthetics increase the $[Ca^{2+}]_i$ in hippocampal [16,21], cerebrocortical [16], cultured mouse embryonic cortical neurons [7] and dorsal root ganglion neurons [11]. The same effect was also observed with halothane and isoflurane in a transformed rat pituitary cell line (GH_3) [13] and with halothane in a cholinergic cell line derived from the fusion of neuroblastoma cell line with medial-septal neurons (SN56) [12]. However, the mechanisms by which these agents increase the $[Ca^{2+}]_i$ are still a matter of debate. Many of the studies mentioned above showed that the increase on $[Ca^{2+}]_i$ occurs due to a release of calcium from intracellular stores, rather than a Ca^{2+} influx from the extracellular medium.

It has been reported that halothane increased $[Ca^{2+}]_i$ in cholinergic cells SN56 and this effect was blocked by dantrolene, an inhibitor of calcium release from ryanodine-sensitive calcium stores [12]. However, there are few studies evaluating the effect of sevoflurane on $[Ca^{2+}]_i$ in neurons [23,30]. In the

* Corresponding author. Tel.: +55 31 3248 9759; fax: +55 31 3248 9760.
E-mail address: renatogomez2000@yahoo.com.br (R.S. Gomez).

0361-9230/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.brainresbull.2005.11.016

present report we have examined the effects of sevoflurane on $[Ca^{2+}]_i$ from a cholinergic cell line (SN56).

2. Materials and methods

2.1. Cell culture

The SN56 cells were a generous gift from Professor Bruce Wainer (Department of Pathology, Emory University School of Medicine, Atlanta, GA, USA). The cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated bovine calf serum, 1% penicillin/streptomycin, 2 mM L-glutamine in 50-ml culture bottles in a 5% CO_2 atmosphere at 37 °C. The SN56 cells were differentiated in the same medium as above lacking fetal bovine serum and supplemented with 1 mM of dibutyril-cyclic-AMP for at least 2 days. The medium was changed every two days, except during the differentiation period, when it was changed every 24 h.

2.2. $[Ca^{2+}]_i$ measurement

Experiments were performed at room temperature (20–25 °C) essentially as previously described [12]. Cells on coverslips were pre-incubated for 1 h in Hepes-buffered salt solution (HBSS) containing in mM: 124 NaCl, 4 KCl, 2 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose, 25 Hepes, pH 7.4 adjusted with NaOH and 10 μM Fluo-4 AM. The coverslips were washed in dye-free HBSS, then transferred to a custom holder in which the coverslips formed the bottom of a 400 μl bath that was perfused continuously (3 ml/min). Imaging was performed with a Bio-Rad MRC 1024 laser scanning confocal system running the software Time course coupled to a Zeiss microscope (Axiovert 100) with a water immersion objective (40×, 1.2 NA). Fluo-4 AM was imaged by excitation with a 488 nm argon laser line and light emitted was collected with a band pass filter (522/35). On the experiments with dantrolene or BAPTA-AM, these drugs were added to SN56 cells 30 min after starting the loading with fluo-4.

2.3. Chemical reagents

Fluo-4 acetoxymethyl ester (fluo-4 AM) was obtained from Molecular Probes (Eugene, OR, USA). Dulbecco's modified Eagle's medium (DMEM), dantrolene, carbachol and others reagents were obtained from Sigma (St Louis, MO, USA). Cyclopiazonic acid and dibutyril-cyclic-AMP were obtained from Alomone (Israel). Xestospongin D was obtained from Calbiochem (San Diego, CA, USA). The *n*-heptane chromatography grade was obtained from Merck Darmstadt (Darmstadt, Germany). All other chemicals and reagents were of analytical grade and were obtained from the usual commercial sources.

2.4. Administration of sevoflurane

Solutions of sevoflurane were prepared prior to each experimental session. The HBSS (10 ml) was equilibrated with liquid anesthetic at room temperature (20°–25 °C) in a capped air-tight syringe for 30 min. Subsequently, the coverslips were perfused continuously by the anesthetic solution. The sevoflurane/HBSS flow through the coverslips was kept constant in all experiments (3 ml/min) in order to minimize differences due to loss of sevoflurane during perfusion. The concentration of sevoflurane in the perfused HBSS was measured by gas chromatography. Sevoflurane extraction from the HBSS was performed by *n*-heptane as previously described [20]. Briefly, 500 μl of the HBSS containing sevoflurane were taken at the beginning and at the end of the perfusion procedure. These aliquots were transferred into 1.5-ml vials containing 1.0 ml of *n*-heptane chromatography grade. This biphasic system was shaken for a period of 5 min and allowed to settle for a minimum of 10 min. Analysis of sevoflurane in the *n*-heptane phase was performed by gas chromatography using a Hewlett Packard Series II-5890 gas chromatograph equipped with a Hewlett Packard SE-30 capillary column, 35 mm × 0.20 mm with a film thickness of 0.33 μm and a flame ionization detector. Volatile anesthetic concentrations were reported in μM concentrations. Measurements at the beginning and at the end of the perfusion showed a negligible loss of anesthetic.

2.5. Data analysis

The values of fluorescence were reported as the mean values ± S.E.M. from cells studied in several experiments and were normalized by subtracting baseline values. The variation on $[Ca^{2+}]_i$ was plotted in arbitrary units (a.u.). Statistical analysis between the means was examined by Student–Newman–Keuls test with a significance of $P < 0.05$.

3. Results

In the first set of experiments, we tested whether sevoflurane induces changes on $[Ca^{2+}]_i$ from SN56 cells. The cells were loaded with the calcium indicator fluo-4 AM and thereafter, they were perfused with HBSS lacking calcium containing 2.0 mM EGTA. After the baseline of fluorescence intensity was reached, sevoflurane was added, at different concentrations (30, 120, 240 and 480 μM), during a 3 min perfusion period. Fig. 1A–D shows that sevoflurane induced a significant increase on fluo-4 fluorescence in a dose-dependent manner ($P < 0.05$). Sevoflurane induced the same level of fluorescence when the cells were perfused with HBSS containing calcium (2.0 mM) (data not shown), suggesting that the sevoflurane effect on $[Ca^{2+}]_i$ was not dependent on extracellular calcium.

In the second set of experiments we tested the hypothesis that the increase on $[Ca^{2+}]_i$ induced by sevoflurane involved the participation of the intracellular calcium and thus, we performed experiments using the intracellular calcium chelator BAPTA-AM [1,17]. SN56 cells were pre-incubated with this agent for 30 min and then perfused with sevoflurane (480 μM) (Fig. 2). We observed that in the presence of BAPTA-AM (10 μM), the sevoflurane-induced increase on $[Ca^{2+}]_i$ in SN56 cells was abolished. This result suggests that the $[Ca^{2+}]_i$ increase induced by sevoflurane is dependent on calcium release from intracellular stores.

We next investigated the participation of endoplasmic reticulum (ER) on this process. To address this point, fluo-4 loaded cells were exposed to 20 μM cyclopiazonic acid, an inhibitor of sarco/endoplasmic reticulum calcium ATPase (SERCA) [3,4], and after 3 min, they were immediately perfused with sevoflurane. Fig. 3 shows that cyclopiazonic acid increases the fluorescence in SN56 cells (first arrow) that returned to the basal level after 3 min. Thereafter, the addition of sevoflurane (480 μM) had no significant effect on $[Ca^{2+}]_i$ from SN56 cells (second arrow) ($P < 0.05$). The cells were then perfused with HBSS containing calcium for 10 min in order to refill the ER stores. They were then challenged with a new perfusion with sevoflurane (480 μM). At this condition, we observed a pronounced increase on $[Ca^{2+}]_i$ (third arrow) that was similar with that obtained when the cells were perfused with the anesthetic alone (Fig. 1), suggesting the involvement of ER on the sevoflurane-induced increase on $[Ca^{2+}]_i$ from SN56 cells.

Considering that the intracellular calcium release from ER occurs through IP_3 and ryanodine receptors, we used pharmacological tools to silence those receptors and investigate if they are involved in the sevoflurane effect on $[Ca^{2+}]_i$. Fig. 4 shows the effect of dantrolene, a blocker of Ca^{2+} release from ryanodine receptor [24,25,29]. After a pre-incubation period of 30 min with