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Effect of Sevoflurane on Ca^{2+} Mobilization in Madin–Darby Canine Kidney Cells

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ABSTRACT. We investigated the effect of the volatile anesthetic sevoflurane on Ca^{2+} signaling in Madin–Darby canine kidney (MDCK) cells by using the fluorescent dye fura-2/AM (1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*,*N*-tetraacetic acid pentaacetoxymethyl ester) as the Ca^{2+} indicator. At a concentration of 0.15 mM, sevoflurane did not alter basal cytosolic free calcium concentration ($[Ca^{2+}]_i$); however, at concentrations of 0.45–0.6 mM, sevoflurane did elevate $[Ca^{2+}]_i$, mainly by releasing Ca^{2+} from the endoplasmic reticulum (ER) store. Sevoflurane (0.15 mM) did not change either the $[Ca^{2+}]_i$ peak evoked by high doses of ATP or UTP or inhibition of the ER Ca^{2+} pump, although it did significantly slow down the decay of the $[Ca^{2+}]_i$ rise. Lastly, sevoflurane inhibited the capacitative Ca^{2+} entry and Mn^{2+} quench of fura-2 fluorescence induced by Ca^{2+}-mobilizing ligands. BIOCHEM PHARMACOL **59**;4:393–400, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. sevoflurane; anesthetics; ATP; calcium, intracellular; fura-2; MDCK cells

Sevoflurane is a polyfluorinated methyl isopropyl ether inhalation general anesthetic which provides for a rapid and smooth induction of, and recovery from, anesthesia. These features, combined with its favorable cardiovascular profile, make it the agent of choice for inhalation induction in adult and pediatric anesthesia [1]. It is thought that the key targets of general anesthetic action at the molecular level are protein receptors rather than lipid membranes [2]. However, the precise cellular mechanisms underlying the action of general anesthetics remain unclear [1]. Sevoflurane has been shown to exert some effects at the cellular levels in several cell types. It was reported that sevoflurane altered inward rectifier potassium current in guinea pig ventricular cardiomyocytes [3], interacted with the nicotinic acetylcholine receptor in mouse myotubes [4] and glycine receptors in *Xenopus* oocytes [5], and potentiated γ-aminobutyric acid-induced chloride current in CA1 pyramidal neurons [6]. Alterations in the level of $[Ca^{2+}]_i$[‖] are closely coupled to many physiological responses in essentially all types of cells [7, 8]. Sevoflurane has been shown to depress cardiac and smooth muscle Ca^{2+} currents [9, 10], inhibit the plasma membrane Ca^{2+} pump leading to a prolonged Ca^{2+} recovery time in response to *N*-methyl-D-aspartic acid stimulation in neuronal cells [11], and to activate release of Ca^{2+} from non-mitochondrial stores in rat hepatocytes [12].

In this study, we examined the effects of sevoflurane on several aspects of Ca^{2+} signaling in MDCK cells: (1) basal $[Ca^{2+}]_i$; (2) $[Ca^{2+}]_i$ rises induced by activation of inositol 1,4,5-triphosphate formation or inhibition of the ER Ca^{2+} pump; and (3) Ca^{2+} influx induced by Ca^{2+}-mobilizing ligands.

MATERIALS AND METHODS

Cell Culture

MDCK cells obtained from the American Type Culture Collection (CRL-6253) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37° in 5% CO_2-containing humidified air.

Solutions

Ca^{2+} medium (pH 7.4) contained (in mM): NaCl 140; KCl 5; $MgCl_2$ 1; $CaCl_2$ 1.8; HEPES 10; glucose 5. Ca^{2+}-free medium contained no Ca^{2+} plus 0.1 mM EGTA (calculated $[Ca^{2+}] < 0.1$ nM). The experimental solution contained ≤0.1% of vehicle (DMSO or ethanol), which did not affect $[Ca^{2+}]_i$ ($N = 3$).

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‖ *Abbreviations:* $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; ER, endoplasmic reticulum; fura-2/AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*,*N*-tetraacetic acid pentaacetoxymethyl ester; MDCK cells, Madin–Darby canine kidney cells; BHQ, 2,5-di-*tert*-butylhydroquinone; and CCE, capacitative Ca^{2+} entry.

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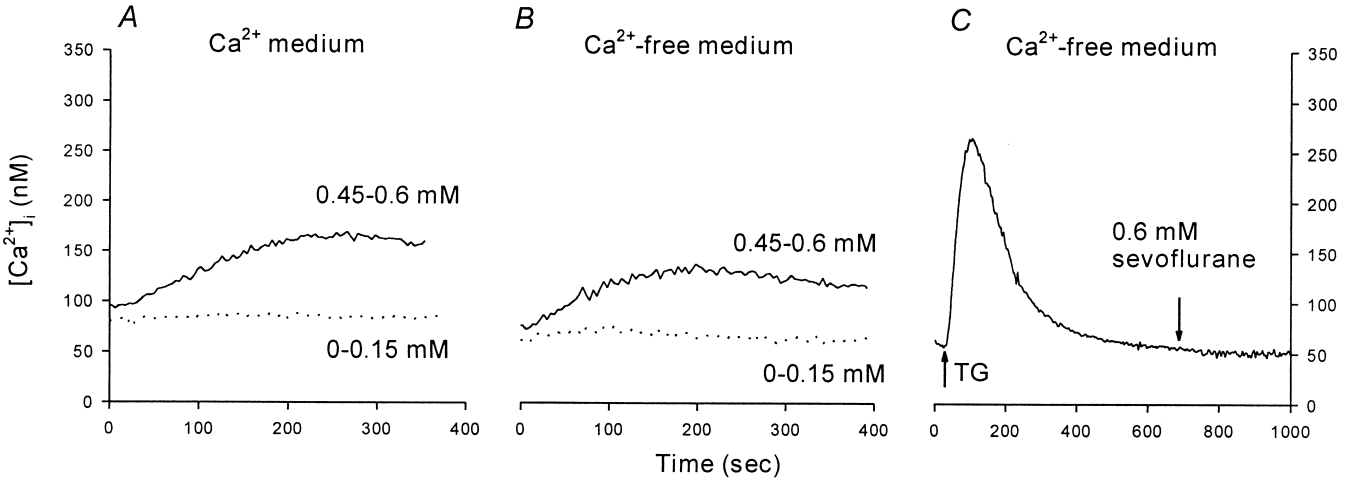

FIG. 1. (A) Effect of sevoflurane on $[Ca^{2+}]_i$ in Ca^{2+} medium. Sevoflurane was added at 0, 0.15, 0.45, and 0.6 mM, respectively. See Methods for details. (B) Similar to A except that the external medium contained no added Ca^{2+} plus 0.1 mM EGTA. (C) Effect of depletion of the ER Ca^{2+} store with thapsigargin on the sevoflurane-induced $[Ca^{2+}]_i$ rise. In Ca^{2+}-free medium, thapsigargin (TG, 1 μM) was added at 30 sec, followed by 0.6 mM sevoflurane at 700 sec. All traces are typical of 5–6 paired experiments.

Preparation of Sevoflurane Solution

An aliquot of pure sevoflurane solution was added to Ca^{2+} medium or Ca^{2+}-free medium, and the mixture was vigorously mixed in a sealed glass bottle for 10 min immediately before the experiment. The experiment was started by adding 0.1 mL of cell suspension to 0.9 mL of the mixture in a cuvette with continuous stirring so that the final sevoflurane concentration in the experimental solution was 0.15 mM, unless otherwise indicated. According to Dobkin *et al.* [13], 0.5 and 2 vol% of sevoflurane at 22° in the normal saline was equivalent to approximately 0.15 and 0.6 mM, respectively. The concentration of sevoflurane in our experimental solutions was checked with gas chromatography.

Optical Measurements of $[Ca^{2+}]_i$

Trypsinized cells (10^6/mL) were loaded with the ester form of fura-2, fura-2/AM (2 μM), for 30 min at 22° in Dulbecco's modified Eagle's medium. Cells were washed and resuspended in Ca^{2+} medium and thereafter washed every hour during experiments to minimize extracellular dye. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (22°) with continuous stirring; the cuvette contained 1 mL of Ca^{2+} medium and 0.5 million cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer (Japan) by continuously recording excitation signals at 340 and 380 nm and the emission signal at 510 nm at 1-sec intervals. Maximum and minimum fluorescences were obtained by adding Triton-100 (0.1%) and EGTA (20 mM) sequentially at the end of an experiment. The ratio of excitation signals at 340 and 380 nm was used to calculate $[Ca^{2+}]_i$ as described previously [14], assuming a K_d of 155 nM. Mn^{2+} quench experiments were performed in Ca^{2+} medium containing 50 μM $MnCl_2$ by recording the excitation signals at 360 nm (the isosbestic point for fura-2) and the emission signal at 510 nm. We previously found that trypsinized cells yielded qualitatively similar results in response to ATP, UTP, and bradykinin as cells attached to coverslips [15–17]. We decided to use trypsinized cells because this procedure is easier and less time-consuming.

Chemical Reagents

The reagents for cell culture were from Gibco. Fura-2/AM was from Molecular Probes. Sevoflurane was from Abbott Laboratories Ltd. and BHQ from Biomol. Other reagents were from Sigma.

Statistical Analysis

All values are reported as means ± SE of 5–6 paired experiments. Statistical comparisons were determined by using Student's paired *t*-test, and significance was accepted when $P < 0.05$.

RESULTS

Effect of Sevoflurane on Basal $[Ca^{2+}]_i$

Figure 1A shows that sevoflurane (0.45–0.6 mM) induced a gradual rise in $[Ca^{2+}]_i$ which peaked at 151 ± 12 nM (N = 6) followed by a sustained plateau phase. Sevoflurane had no effect at 0.15 mM. This sevoflurane-induced rise in $[Ca^{2+}]_i$ was partly reduced by removal of external Ca^{2+} by 13 ± 2% (131 ± 9 nM vs 151 ± 12; N = 5; $P < 0.05$) at peak height, and the area under the curve was reduced by 10 ± 3% (N = 5; $P < 0.05$) (Fig. 1B). We examined the internal Ca^{2+} source(s) for the sevoflurane response. Fig.

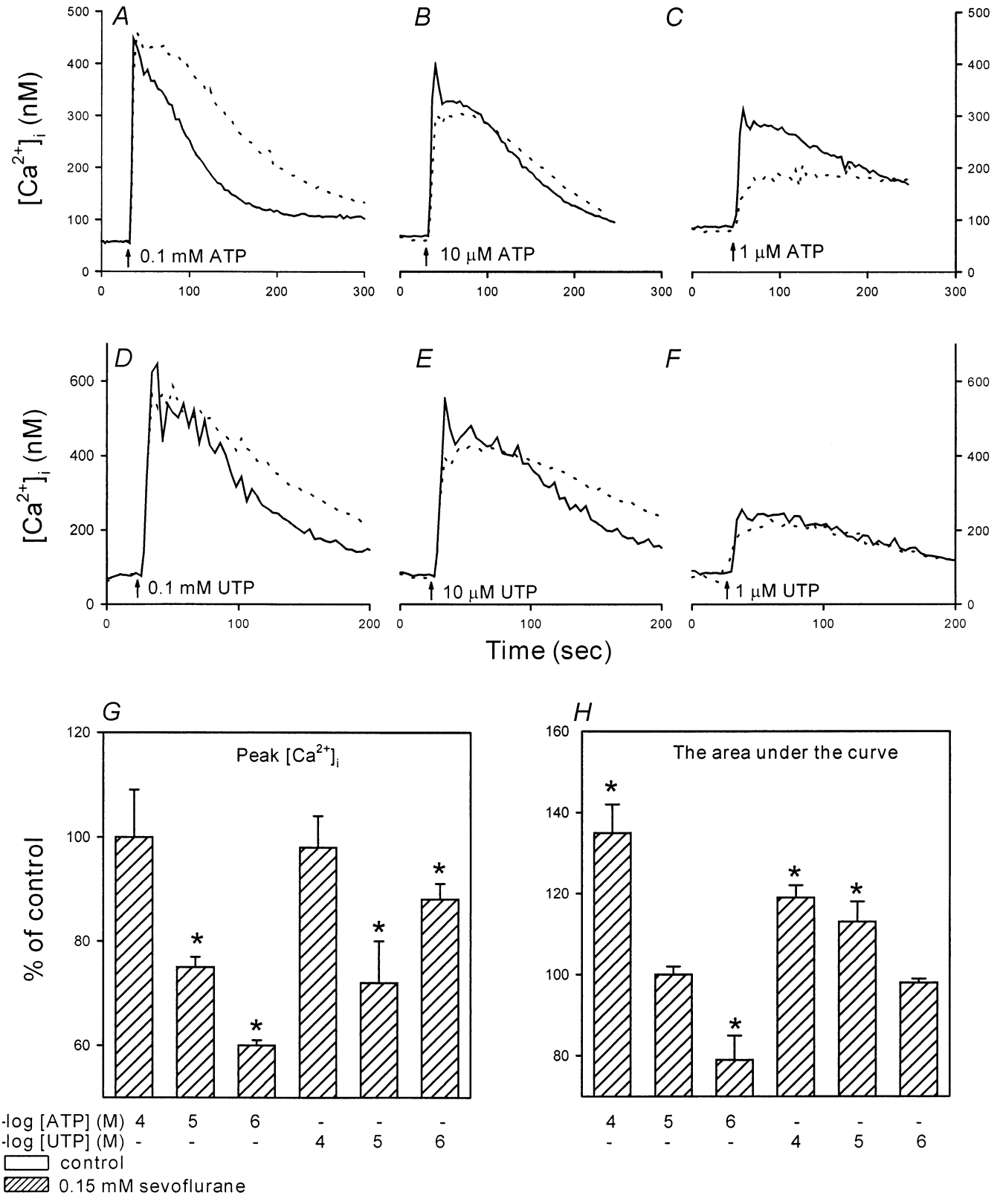

FIG. 2. (A–F) Effect of sevoflurane (0.15 mM; *dashed traces*) on ATP- or UTP-evoked $[Ca^{2+}]_i$ rise. *Solid traces*: control without sevoflurane. The experiments were performed in Ca^{2+} medium. In the sevoflurane group, the medium was equilibrated with sevoflurane ~30 sec prior to addition of the nucleotide. All traces are typical of 5–6 paired experiments. (G and H) Bar graphs summarizing the effect of sevoflurane on the peak value (G) and the area under the curve (H) of the nucleotide-evoked $[Ca^{2+}]_i$ rise as shown in A–F. The y-axis represents percentage of control. The error bars represent means ± SE of 5–6 paired experiments. *$P < 0.05$.

1C shows that in the absence of external Ca^{2+}, the ER Ca^{2+} pump inhibitor thapsigargin (1 μM) [18] induced a $[Ca^{2+}]_i$ rise with a peak value of 262 ± 18 nM (N = 6), which returned to baseline in 10 min. Subsequent addition of 0.6 mM sevoflurane failed to increase $[Ca^{2+}]_i$, suggesting that thapsigargin depleted the sevoflurane-sensitive internal Ca^{2+} store. Because sevoflurane at a concentration of 0.15 mM did not alter basal $[Ca^{2+}]_i$, this concentration was used in the subsequent experiments.

Effect of Sevoflurane on the Ca^{2+} Signals Induced by P2 Purinoceptor Agonists

Figure 2, A–F shows that ATP (or UTP) at 0.1 mM, 10 μM, or 1 μM induced a dose-dependent $[Ca^{2+}]_i$ rise (*solid traces*). Sevoflurane enlarged the $[Ca^{2+}]_i$ transient induced by 0.1 mM ATP (or UTP) by elevating the decay phase without altering the peak value (*dotted trace*). At lower doses of ATP or UTP (1 or 10 μM), sevoflurane reduced the peak value, and the effect on the decay phase became smaller. Sevoflurane had a similar effect on the $[Ca^{2+}]_i$ rise induced by the non-hydrolyzable ATP analogue ATP γ S (not shown). Figure 2, G and H summarizes the effect of sevoflurane on the peak $[Ca^{2+}]_i$ and the area under the curve of the $[Ca^{2+}]_i$ rises induced by the nucleotides.

Effect of Sevoflurane on $[Ca^{2+}]_i$ Rises Induced by the ER Ca^{2+} Pump Inhibitors

We applied three ER Ca^{2+} pump inhibitors, i.e. thapsigargin, cyclopiazonic acid (CPA), and BHQ [18–21], to deplete the ER Ca^{2+} store. Figure 3, A–C shows that in Ca^{2+}-free medium these agents induced a $[Ca^{2+}]_i$ rise consisting of a gradual rise and a slow decay (control; *solid traces*). Sevoflurane (0.15 mM) did not alter the rising phase nor the peak height of the $[Ca^{2+}]_i$ transient, but did significantly elevate the decay phase (*dotted traces*). Figure 3D summarizes the effects of sevoflurane on the area under the curve of the $[Ca^{2+}]_i$ rises induced by these agents.

Effect of Sevoflurane on Capacitative Ca^{2+} Entry (CCE)

We have previously shown that ATP, UTP, and BHQ [15, 16, 21] activate CCE, which is a Ca^{2+} entry process induced by depletion of internal Ca^{2+} stores [22]. In our experiments, CCE was induced by first depleting the ER Ca^{2+} stores with the ligand in Ca^{2+}-free medium followed by addition of 5 mM $CaCl_2$. Figure 4, A–C depicts the ATP-, UTP-, or BHQ-induced $[Ca^{2+}]_i$ rise, which returned to baseline in 200–400 sec (phase 1; *trace a*). Subsequently added $CaCl_2$ induced a $[Ca^{2+}]_i$ rise which was significantly greater than control (without ligand pretreatment, *trace c* in Fig. 4A). Thus, this $[Ca^{2+}]_i$ rise is most likely due to CCE. In the presence of 0.15 mM sevoflurane, the $[Ca^{2+}]_i$ rise was significantly enhanced (phase 1; *trace b*) compared with control (in the absence of sevoflurane; *trace a*). In contrast,

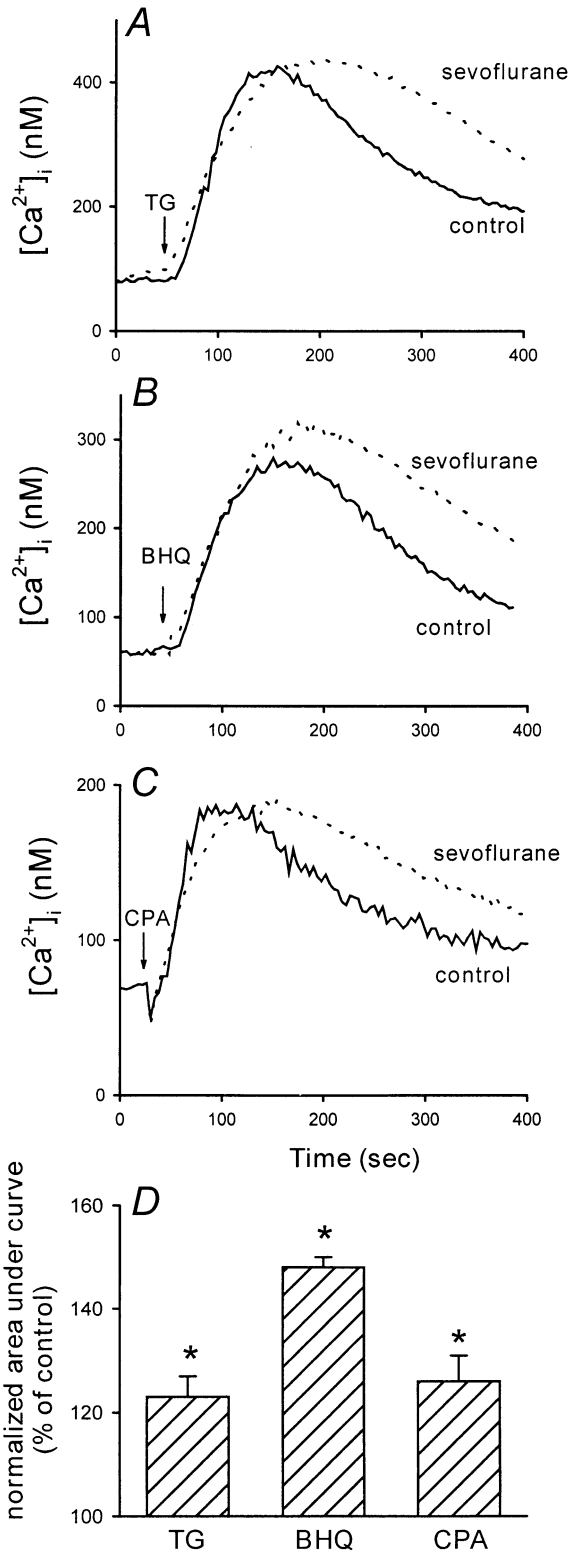

FIG. 3. Effects of sevoflurane on the $[Ca^{2+}]_i$ rises induced by (A) thapsigargin (TG; 1 μM), (B) BHQ (0.1 mM), or (C) cyclopiazonic acid (CPA; 0.1 mM). The experiments were performed in Ca^{2+}-free medium. *Dashed traces*: 0.15 mM sevoflurane group. *Solid traces*: control without sevoflurane. All traces are typical of 5–6 paired experiments. (D) Bar graph summarizing the effect of sevoflurane on the area under curve of the $[Ca^{2+}]_i$ rise. The y-axis represents percentage of control. The error bars represent means ± SE of 5–6 paired experiments. *P < 0.05.

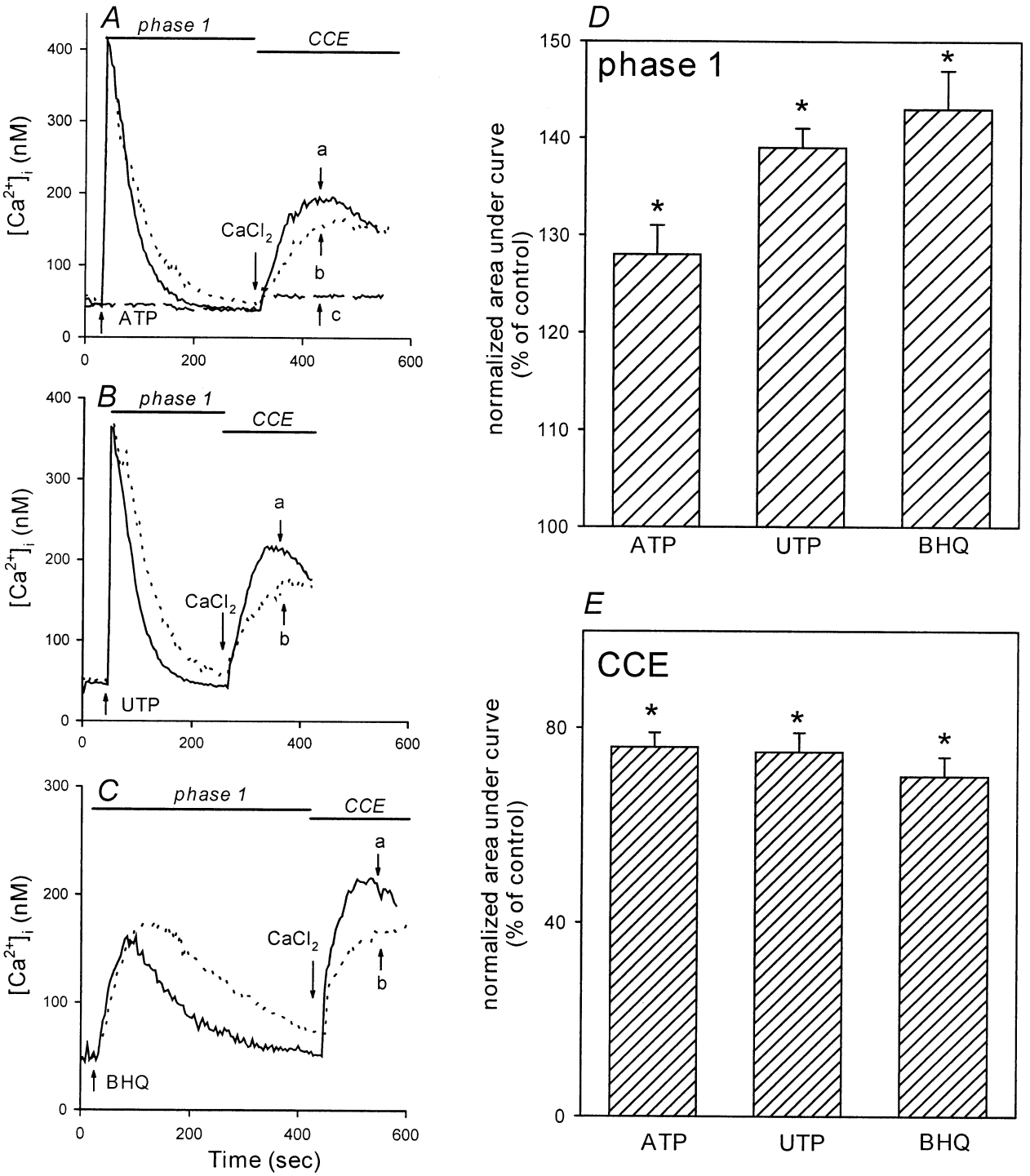

FIG. 4. Effect of sevoflurane on capacitative Ca^{2+} entry. ATP (A), UTP (B), or BHQ (C) was added at 30 sec in Ca^{2+}-free medium. This induced a $[Ca^{2+}]_i$ rise which is referred to as phase 1. $CaCl_2$ (5 mM) was subsequently added as indicated and induced a $[Ca^{2+}]_i$ rise which is referred to as CCE. *Trace b*: 0.15 mM sevoflurane group. *Trace a*: control without sevoflurane. In (A), *trace c* represents control effect of addition of $CaCl_2$ in the presence or absence of sevoflurane without pretreatment with ATP. All traces are typical of 5–6 paired experiments. (D and E) Bar graphs summarizing the effect of sevoflurane on the normalized area under the curve of phase 1 (D) and CCE (E) of the $[Ca^{2+}]_i$ rise evoked by 0.1 mM ATP, UTP, or BHQ as shown in A–C. The y-axis represents percentage of control. The error bars represent means ± SE of 5–6 paired experiments. *$P < 0.05$.

upon addition of $CaCl_2$, the CCE induced by these ligands was significantly less than control both in the peak value and the area under the curve (*trace b* vs *trace a*). The effect of adding $CaCl_2$ alone in the presence of sevoflurane was indistinguishable from *trace c* in Fig. 4A, and thus was not shown. Figure 4, D and E summarizes the effect of sevoflurane on the area under the curve of phase 1 and CCE, respectively.

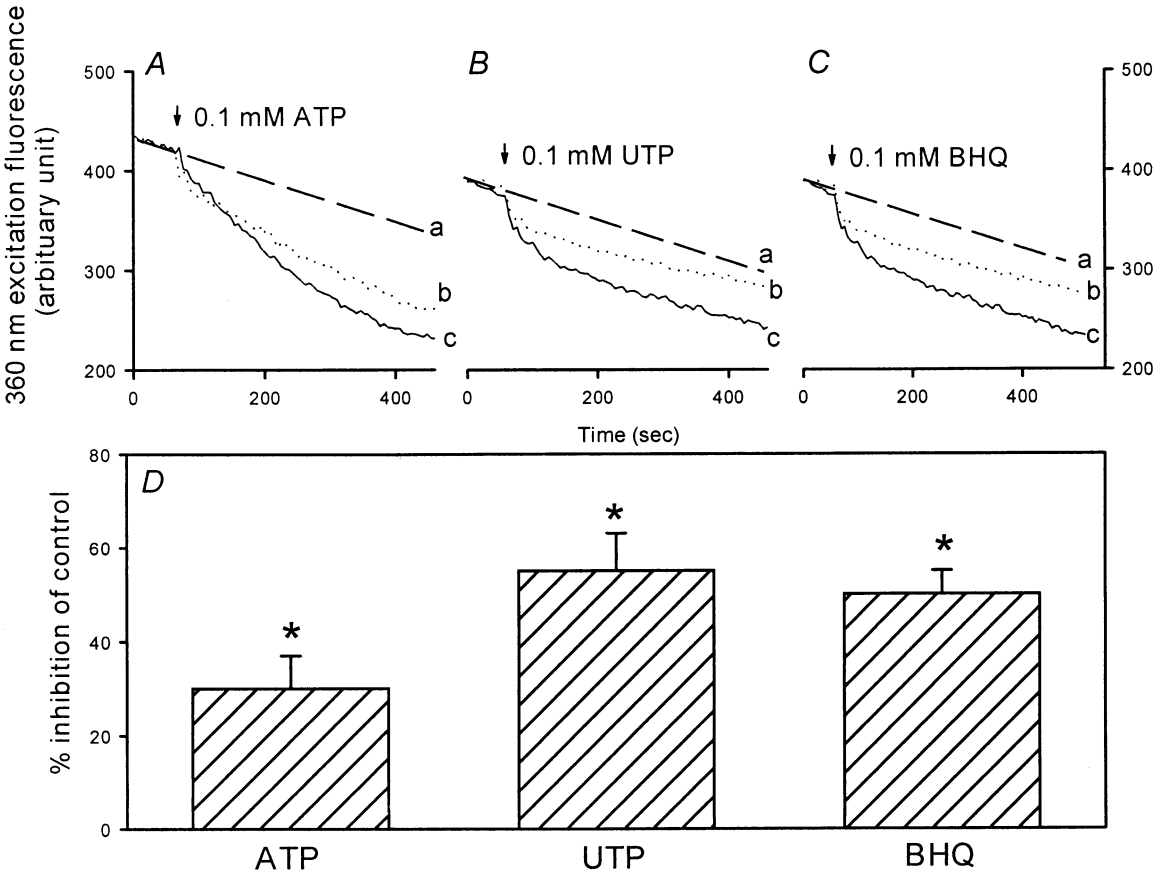

FIG. 5. Effect of sevoflurane on Mn^{2+} entry induced by ATP (A), UTP (B), or BHQ (C). The experiments were performed in Ca^{2+} medium containing 50 μM $MnCl_2$. See Methods for details. *Trace a*: control effect of addition of $MnCl_2$ alone without addition of sevoflurane or the ligand. *Trace b*: the medium contained 0.15 mM sevoflurane, and the ligand was added as indicated. *Trace c*: effect of the stimulant alone without addition of sevoflurane. All traces are typical of 5–6 paired experiments. (D) Bar graph summarizing the effect of sevoflurane on Mn^{2+} entry induced by ATP, UTP, or BHQ. The y-axis presents percentage inhibition of control, which is the difference between the area under the curve of *trace a* and *trace c*. The error bars represent means ± SE of 5–6 paired experiments. *$P < 0.05$.

Effect of Sevoflurane on Ca^{2+} Influx

Mn^{2+} enters cells through similar pathways as Ca^{2+}, but quenches fura-2 fluorescence at all excitation wavelengths [23]. Thus, Ca^{2+} influx can be reported by the Mn^{2+} quench of fura-2 fluorescence at the Ca^{2+}-insensitive excitation wavelength of 360 nm and emission wavelength of 510 nm. Figure 5, A–C shows that in the presence of 50 μM $MnCl_2$, ATP, UTP, and BHQ all induced a significant decrease in the 360-nm excitation signal (*trace c*) compared with the effect of addition of $MnCl_2$ alone (*trace a*). This is consistent with the observation in Fig. 4 that these agents induced CCE. Sevoflurane (0.15 mM) inhibited the ligand-induced Mn^{2+} quench (*trace b*). Figure 5D shows the effect of sevoflurane by computing the area under the trace and is expressed as percentage inhibition of control.

DISCUSSION

In this study, we have investigated the effect of sevoflurane on Ca^{2+} signaling in MDCK cells. We found that sevoflurane at 0.45–0.6 mM induced a $[Ca^{2+}]_i$ rise by triggering both external Ca^{2+} influx and internal Ca^{2+} release, since removal of external Ca^{2+} only partly inhibited the $[Ca^{2+}]_i$ rise. We also observed that at a concentration of 0.15 mM sevoflurane did not alter basal $[Ca^{2+}]_i$. Among the other volatile anesthetics, isoflurane was shown to cause external Ca^{2+} influx and internal Ca^{2+} release in vascular smooth muscle [24], and that halothane was shown to release internal Ca^{2+} in hepatocytes [25, 26]. Additionally, we found that the internal Ca^{2+} source for the sevoflurane-induced $[Ca^{2+}]_i$ rise was the ER, since after depletion of this store by thapsigargin in Ca^{2+}-free medium, sevolfurane did not increase $[Ca^{2+}]_i$. The mechanism underlying the sevoflurane-induced Ca^{2+} influx is unclear, but might take place via CCE or non-selective cation channels, which are known to be the major Ca^{2+} entry pathways in MDCK cells [15–17, 20, 21].

Because sevoflurane has been shown to interact with protein receptors in the plasma membrane [1, 2], we examined its effects on the Ca^{2+} signals induced by P2

purinoceptor agonists. Sevoflurane (0.15 mM) affected the agonist-induced $[Ca^{2+}]_i$ rise in a manner dependent on the dose of the agonist and potentiated the $[Ca^{2+}]_i$ transient induced by a high concentration of agonists (0.1 mM) without altering the peak value. In neural cells, sevoflurane, isoflurane, and halothane have been shown to inhibit plasmalemmal Ca^{2+} pumps, leading to delayed Ca^{2+} efflux and enlarged $[Ca^{2+}]_i$ transients in response to *N*-methyl-D-aspartic acid [11]. Additionally, halothane and isoflurane were found to inhibit the Ca^{2+} pump in rat brain synaptic plasma membranes [27], and halothane was reported to inhibit the pump in C6 glioma cells [28]. We showed recently that La^{3+}, a blocker of the plasmalemmal Ca^{2+} pump [29], potentiated the $[Ca^{2+}]_i$ rise induced by bradykinin and thapsigargin in a manner similar to sevoflurane's action on the nucleotide-induced $[Ca^{2+}]_i$ rise. This interpretation is supported by the observation that sevoflurane also potentiated the $[Ca^{2+}]_i$ rise induced by inhibition of the ER Ca^{2+} pump by thapsigargin, cyclopiazonic acid, or BHQ. Another interpretation for sevoflurane's effect is that it enhances the ligand-induced Ca^{2+} influx. Our results suggested this to be unlikely, because sevoflurane inhibited ligand-induced Ca^{2+} influx (Figs. 4 and 5), and its effect was observed in the absence of external Ca^{2+} (Fig. 4). In contrast, sevoflurane reduced the peak value of the $[Ca^{2+}]_i$ rise induced by a low dose of nucleotides (1 μM) without altering the plateau phase. Sevoflurane apparently did not act via inhibiting CCE, because at 1 μM these nucleotides did not induce significant CCE (not shown). One possible explanation is that sevolflurane inhibited P2 purinoceptors, and the inhibition could be overcome by a maximum stimulation of the receptors. In endothelial cells, sevoflurane was shown to inhibit the bradykinin-induced $[Ca^{2+}]_i$ rise [30]. Additionally, sevoflurane was demonstrated to interfere with nicotinic receptors in myotubes [4] and glycine receptors in oocytes [5]. Other anesthetics such as halothane and enflurane (but not isoflurane) were shown to inhibit the ATP-induced $[Ca^{2+}]_i$ rise in endothelial cells [31].

In cortical neurons maintained at 37°, sevoflurane, isoflurane, and halothane inhibited plasmalemmal Ca^{2+} pumps, leading to a slower decay of the $[Ca^{2+}]_i$ rise induced by *N*-methyl-D-aspartic acid and an increased basal and peak $[Ca^{2+}]_i$. However, at 21° these effects could not be observed [11]. Our data suggest that sevoflurane potentiated the ligand-induced $[Ca^{2+}]_i$ rise at 22°. This discrepancy might be due to differences in cell type. Halothane and isoflurane were shown to inhibit bradkinin-induced CCE in endothelial cells [32]. Our results suggest that sevoflurane partly inhibited the CCE induced by either P2 purinoceptor activation or inhibition of the ER Ca^{2+} pump. Consistently, the Mn^{2+} entry data suggest that sevoflurane inhibited the ligand-activated Ca^{2+} influx. The minimum alveolar concentration (MAC) of sevoflurane for anesthesia is known to be 0.3–0.4 mM in the blood phase [33, 34]. Thus, the concentrations of sevoflurane (0.15–0.6 mM) used in this study were of physiological relevance.

In conclusion, in this study we have examined the effect of sevoflurane on Ca^{2+} signaling in a renal cell. We found that sevoflurane altered both basal $[Ca^{2+}]_i$ and the ligand-induced $[Ca^{2+}]_i$ rise. Our data may contribute to a greater understanding of how sevoflurane acts as an anesthetic at the cellular level.

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