

# Differential Inhibition of Neuronal $\text{Na}^+$ - $\text{Ca}^{2+}$ Exchange versus Store-operated $\text{Ca}^{2+}$ Channels by Volatile Anesthetics in Pheochromocytoma (PC12) Cells

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**Background:**  $\text{Ca}^{2+}$  influx is a key component of neuronal intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) regulation. The authors hypothesized that volatile anesthetic inhibition of neuronal activity is mediated by inhibition of  $\text{Ca}^{2+}$  influx via two major mechanisms: plasma membrane  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchange (NCX) and the novel mechanism of  $\text{Ca}^{2+}$  influx triggered by endoplasmic reticulum  $\text{Ca}^{2+}$  depletion (store-operated  $\text{Ca}^{2+}$  channels [SOCCs]).

**Methods:** Differentiated rat pheochromocytoma cells loaded with the  $\text{Ca}^{2+}$  indicator fura-2 were  $\text{Na}^+$ -loaded with 0  $\text{Ca}^{2+}$ , 145 mM  $\text{Na}^+$  Tyrode's and 5  $\mu\text{M}$  cyclopiazonic acid plus 10  $\mu\text{M}$  ryanodine (functionally isolating plasma membrane). Influx-mode NCX was rapidly reactivated by 0  $\text{Na}^+$  and 2.5 mM  $\text{Ca}^{2+}$ . The protocol was repeated in the presence of volatile anesthetics (0.5–1.5 minimum alveolar concentration [MAC] halothane, isoflurane, or sevoflurane) or other drugs to characterize NCX. To examine SOCCs, endoplasmic reticulum  $\text{Ca}^{2+}$  was depleted by cyclopiazonic acid in 0 extracellular  $\text{Ca}^{2+}$ , and  $\text{Ca}^{2+}$  influx was triggered by rapid reintroduction of extracellular  $\text{Ca}^{2+}$ . The protocol was repeated in the presence of anesthetics or other drugs to characterize SOCCs.

**Results:** Influx via NCX was not inhibited by voltage-gated  $\text{Ca}^{2+}$  channel blockers but was sensitive to NCX inhibitors. Halothane and isoflurane (0.5–1.5 MAC) significantly inhibited NCX ( $P < 0.05$ ; paired comparisons), whereas sevoflurane at less than 1.5 MAC did not inhibit NCX. SOCC-mediated  $\text{Ca}^{2+}$  influx was insensitive to a variety of  $\text{Ca}^{2+}$  channel blockers but was inhibited by  $\text{Ni}^{2+}$ . Such influx was sensitive only to halothane at greater than 1 MAC but not isoflurane or sevoflurane.

**Conclusions:** These data indicate that volatile anesthetics, especially halothane and isoflurane, interfere with neuronal  $[\text{Ca}^{2+}]_i$  regulation by inhibiting NCX but not SOCC-mediated  $\text{Ca}^{2+}$  influx (except high concentrations of halothane).

VOLATILE anesthetics influence  $\text{Ca}^{2+}$  regulation in a number of cell systems. Nowhere are these effects more important than in neuronal cells in the action of volatile anesthetics. Understanding anesthetic effects on intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) regulation is important in at least two ways: (1) Synaptic transmission in itself is modulated by local  $\text{Ca}^{2+}$  concentrations at the presynaptic terminal,<sup>1-5</sup> and (2) global  $\text{Ca}^{2+}$  modulates expression and regulation of several genes that affect neuronal func-

tion, plasticity, and survival.<sup>4,6</sup> Although there is extensive literature on synaptic transmission and volatile anesthetic effects,<sup>7-13</sup> there is relatively little literature on anesthetic effects on specific intracellular mechanisms that modulate  $\text{Ca}^{2+}$  during neuronal activation.

$\text{Ca}^{2+}$  regulation in neuronal cells is complex.<sup>6,14,15</sup>  $\text{Ca}^{2+}$  influx is key to modulation of intracellular processes. Influx itself is known to occur through several mechanisms, including N-type and T-type  $\text{Ca}^{2+}$  channels, nonspecific cation channels, receptor-operated specific and nonspecific channels, and finally  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchange (NCX). In this regard, the bidirectional NCX may play an important role both in  $\text{Ca}^{2+}$  influx and removal of  $\text{Ca}^{2+}$  during neuronal activation and in excitotoxicity.<sup>16,17</sup> Furthermore, NCX-mediated  $\text{Ca}^{2+}$  influx and efflux can modulate synaptic transmission.<sup>18</sup> Given that  $\text{Ca}^{2+}$  transport via NCX is much faster than via voltage-gated channels, NCX may be important in both short-term and long-term modulation of neuronal activation.

As with peripheral tissues, endoplasmic reticulum (ER)  $\text{Ca}^{2+}$  release (and perhaps mitochondrial  $\text{Ca}^{2+}$  as well) contributes to total  $\text{Ca}^{2+}$ , at least at the global level. ER  $\text{Ca}^{2+}$  release is known to occur through both inositol trisphosphate ( $\text{IP}_3$ )<sup>19</sup> and ryanodine receptor (RyR)<sup>20</sup> channels. There is now considerable evidence from different cell types, including neurons, that  $\text{Ca}^{2+}$  influx also occurs through specific store-operated  $\text{Ca}^{2+}$  channels (SOCCs; also termed *capacitative  $\text{Ca}^{2+}$  entry*) in response to ER  $\text{Ca}^{2+}$  depletion, thus allowing for replenishment of intracellular  $\text{Ca}^{2+}$  stores.<sup>21-25</sup> Such influx does not seem to be mediated via voltage-gated or receptor-operated channels.<sup>21</sup> In neuronal cells, SOCC-mediated  $\text{Ca}^{2+}$  influx seems to be present in several different cell types and is involved in modulation of neuronal activity and in synaptic plasticity.<sup>22,26-29</sup> The existence of SOCCs in neurons points to a novel mechanism of neuronal modulation that is yet to be explored fully.

Volatile anesthetics have the potential to influence each and every one of the  $\text{Ca}^{2+}$  regulatory mechanisms mentioned above. Previous studies have used electrophysiologic techniques to determine that anesthetics inhibit neuronal L-type,<sup>30</sup> N-type,<sup>31</sup> and T-type  $\text{Ca}^{2+}$  channels.<sup>32</sup> Whether NCX is affected in neurons is not known. However, in cardiac muscle, we have previously demonstrated that volatile anesthetics inhibit NCX.<sup>33,34</sup> This  $\text{Ca}^{2+}$  regulatory mechanism is particularly important in neurons where fast rates of action potential generation and changes in intracellular  $\text{Ca}^{2+}$  require rapid

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transfer of  $\text{Ca}^{2+}$  in and out of the cell. At the level of the synapse, local densities of influx channels and exchangers likely influence synaptic transmission. Anesthetic inhibition of SOCC-mediated  $\text{Ca}^{2+}$  influx may be particularly important. Previous studies have demonstrated that anesthetics actually deplete ER  $\text{Ca}^{2+}$  stores in different cell types.<sup>35,36</sup> This should normally trigger SOCC-mediated  $\text{Ca}^{2+}$  influx. However, inhibition of this mechanism by anesthetics would maintain the ER in a state of depletion, preventing the normal  $\text{Ca}^{2+}$  response of the neuron to electrical or agonist stimulation. Given the relative novelty of the SOCC mechanism, there are currently no data on anesthetic effects on this mechanism in neurons. However, we have recently demonstrated that clinically relevant concentrations of halothane, isoflurane, and sevoflurane inhibit SOCC-mediated  $\text{Ca}^{2+}$  influx in airway smooth muscle.<sup>37</sup>

In the current study, we examined the effects of clinically relevant concentrations of halothane, isoflurane, and sevoflurane on influx-mode NCX and SOCC-mediated  $\text{Ca}^{2+}$  influx in differentiated rat pheochromocytoma (PC12) cells, which served as an immortalized model of neuronal cells. We hypothesized that volatile anesthetic inhibition of neuronal activity is mediated by inhibition of  $\text{Ca}^{2+}$  influx *via* NCX and the novel mechanism of SOCCs.

## Materials and Methods

### PC12 Cell Culture

PC12 cells (American Type Culture Collection, Manassas, VA) were cultured in incubation flasks with RPM-1640 medium, 5% heat-inactivated fetal bovine serum, 10% heat-inactivated horse serum, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 100 U/ml penicillin at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$ -95% air. At 60–75% confluence, cells were resuspended and seeded on eight-well coverslip-bottom chambers for plating (2- to 3-day incubation). Nerve growth factor (per supplier recommendations) was then used to induce cell differentiation. Serum starvation for growth arrest was performed only for 24 h before experiments. Continued cellular response to agonists and maintained  $[\text{Ca}^{2+}]_i$  concentrations were used as surrogate indicators for lack of apoptotic cell death. Finally, using light microscopy, differentiated PC12 cells were identified morphologically by neurite outgrowth.

### $[\text{Ca}^{2+}]_i$ Imaging

Differentiated PC12 cells were incubated in 2.5  $\mu\text{M}$  fura-2/AM (Molecular Probes, Eugene OR) and visualized using a MetaFluor real-time fluorescence imaging system (Universal Imaging, Downingtown, PA) on a Nikon Diaphot inverted microscope (Fryer Instruments, Edina, MN). Cells were initially perfused with normal Tyrode's

solution containing 145 mM NaCl, 4 mM KCl, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , 10 mM glucose, and 10 mM HEPES (pH 7.4; 25°C). A custom-built fluid-level controller was used to perfuse the cells, which minimized changes in fluid level but allowed for rapid exchange of perfusate (< 300 ms). Images (640 × 480 pixels) were acquired at 30 frames/s at 400× magnification (40×/1.25 oil-immersion lens). The  $[\text{Ca}^{2+}]_i$  responses of 25 cells/chamber were obtained using individual, software-defined regions of interest. Fura-2 levels were calibrated for  $[\text{Ca}^{2+}]_i$  using the technique of Grynkiewicz.<sup>38</sup>

### Volatile Anesthetics

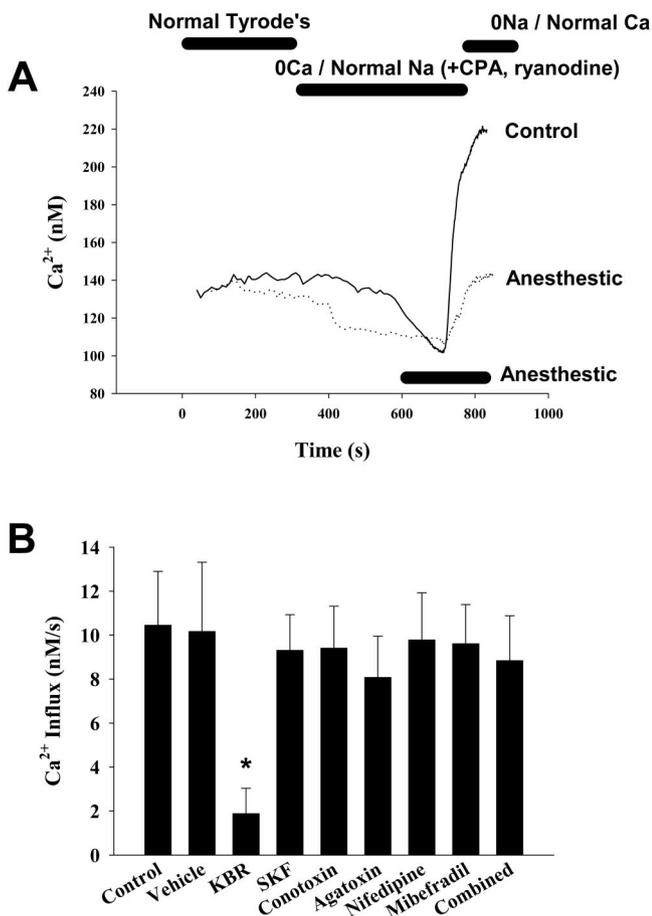
As in previous studies,<sup>37</sup> a calibrated on-line vaporizer was used to add halothane (Wyeth-Ayerst Laboratories, Philadelphia, PA), isoflurane (Abbott Laboratories, Deerfield, IL), and sevoflurane (Abbott) to the aerating gas mixture. Aqueous anesthetic concentrations equivalent to 0.5, 1, and 1.5 minimum alveolar concentration (MAC) at room temperature (25°C) were measured for halothane and isoflurane by gas chromatography and an electron capture detector (Hewlett-Packard 5880A, Palo Alto, CA) and for sevoflurane using a flame ionization detector. Aqueous halothane concentrations equivalent to 1 and 2 MAC were  $0.32 \pm 0.08$  and  $0.50 \pm 0.09$  mm halothane,  $0.32 \pm 0.08$  and  $0.50 \pm 0.09$  mm isoflurane, and  $0.48 \pm 0.06$  and  $0.70 \pm 0.08$  mm sevoflurane, respectively.

### Drugs and Chemicals

All drugs were obtained from Calbiochem/EMD Biosciences (San Diego, CA) unless stated otherwise. Cell culture media were obtained from Hyclone (Logan, UT).

### Effects of Volatile Anesthetics on Influx-mode NCX

The technique for evaluating the effect of volatile anesthetics on influx mode of NCX has been previously published in cardiac muscle<sup>33</sup> and is illustrated in figure 1. PC12 cells were initially perfused with normal Tyrode's, and resting  $[\text{Ca}^{2+}]_i$  concentrations were recorded to ensure cell stability. Stable cells were identified, and regions of interest for  $[\text{Ca}^{2+}]_i$  measurement were outlined. Cells were then  $\text{Na}^+$ -loaded by perfusion with Tyrode's solution containing 0  $\text{Ca}^{2+}$  ( $\text{Ca}^{2+}$ -free Tyrode's with 5 mM EGTA) and normal  $\text{Na}^+$ , 5  $\mu\text{M}$  cyclopiazonic acid (CPA; inhibitor of the sarcoendoplasmic reticulum  $\text{Ca}^{2+}$  adenosine triphosphatase), 10  $\mu\text{M}$  Xestospingon D ( $\text{IP}_3$  receptor channel blocker), and 10  $\mu\text{M}$  ryanodine (RyR channel blocker). Therefore, the ER was effectively inhibited, and the plasma membrane was functionally isolated. After  $\text{Na}^+$ -loading cells for 2 min, perfusion was rapidly switched (< 300 ms) to Tyrode's solution containing 0  $\text{Na}^+$ , normal  $\text{Ca}^{2+}$ , CPA, Xestospingon D, and ryanodine, selectively activating the influx mode of NCX.<sup>33</sup> The rate of increase of  $[\text{Ca}^{2+}]_i$  was measured and served as an index of NCX activity in the



**Fig. 1.** (A) Schematic of the protocol for examining the effect of volatile anesthetics on influx-mode Na<sup>+</sup>-Ca<sup>2+</sup> exchange (NCX) in differentiated rat pheochromocytoma (PC12) cells. After initial wash in normal Tyrode's solution (145 mM Na<sup>+</sup>, 1 mM Ca<sup>2+</sup>), cells were Na<sup>+</sup>-loaded by exposure to 0 Ca<sup>2+</sup> Tyrode's under conditions of blocked endoplasmic reticulum Ca<sup>2+</sup> release (ryanodine) and reuptake (cyclopiazonic acid [CPA]). Intracellular Ca<sup>2+</sup> concentrations decreased slightly due to activation of efflux-mode NCX. NCX-mediated Ca<sup>2+</sup> influx was then selectively activated by rapid reintroduction of extracellular Ca<sup>2+</sup> and simultaneous removal of Na<sup>+</sup>, in the presence or absence of volatile anesthetic (or other drugs). The rate of increase in intracellular Ca<sup>2+</sup> concentrations was used as an index of NCX activity. (B) Characterization of NCX-mediated Ca<sup>2+</sup> influx. Such influx was sensitive to the NCX inhibitor KB-R7943 (KBR) but not to inhibitors of other Ca<sup>2+</sup> influx channels (nifedipine [L type],  $\omega$ -agatoxin IVA [P/Q type],  $\omega$ -conotoxin MVIIA [N type], mibefradil [T type], and SKF-96365 [SKF; receptor-operated channel]). Simultaneous inhibition of different voltage-gated channels (combined) also did not substantially inhibit the observed influx. \* Significant difference from control ( $P < 0.05$ ). Values are presented as mean  $\pm$  SE.

influx mode. After 1 min of measurement, the cells were washed for 10 min with normal Tyrode's solution. Ryanodine is not easily washed out by this technique. However, its presence was not expected to adversely affect subsequent manipulations.

After the wash, the above protocol was repeated with preexposure to 0.5, 1, or 2 MAC volatile anesthetic (halothane, isoflurane, or sevoflurane) before rapid activation of NCX. In control experiments, the protocol was

repeated without volatile anesthetics. The observed Ca<sup>2+</sup> influx was verified to be NCX mediated by repeating the above protocol in a separate set of cells using the NCX inhibitor KB-R7943 (KBR; 10  $\mu$ M). In other cells, the effect of specific voltage-gated Ca<sup>2+</sup> channel inhibitors (the L-type Ca<sup>2+</sup> channel antagonist nifedipine, 1  $\mu$ M; P/Q type Ca<sup>2+</sup> channel inhibitor  $\omega$ -agatoxin IVA, 1 nM; N-type Ca<sup>2+</sup> channel inhibitor  $\omega$ -conotoxin MVIIA, 1 nM; and T-type Ca<sup>2+</sup> channel inhibitor mibefradil, 5  $\mu$ M) and receptor-operated channel inhibitor (SKF-96365, 10  $\mu$ M) on the observed Ca<sup>2+</sup> influx was examined. To eliminate the contribution of voltage-gated channels *per se*, an additional set of experiments was performed in the simultaneous presence of nifedipine, conotoxin, agatoxin, and mibefradil.

#### Store-operated Ca<sup>2+</sup> Influx

The technique for examining SOCC-mediated Ca<sup>2+</sup> influx has also been recently described.<sup>37,39</sup> For these experiments, a Hanks balanced salt solution with or without 2.5 mM Ca<sup>2+</sup> was used. After baseline Ca<sup>2+</sup> concentrations were measured, extracellular Ca<sup>2+</sup> was removed by exposure to 0 Ca<sup>2+</sup> Hanks balanced salt solution (5 mM EGTA). In the continued absence of extracellular Ca<sup>2+</sup>, cells were also exposed to 1  $\mu$ M nifedipine and 10 mM KCl to ensure that L-type Ca<sup>2+</sup> channels were not activated. Cells were then rapidly exposed to 1  $\mu$ M CPA in 0 Ca<sup>2+</sup> Hanks balanced salt solution. This technique passively depletes ER Ca<sup>2+</sup> by preventing reuptake during ongoing Ca<sup>2+</sup> leak from the ER. In addition, Ca<sup>2+</sup> influx is also prevented because no extracellular Ca<sup>2+</sup> is present. When the [Ca<sup>2+</sup>]<sub>i</sub> response to CPA was verified, 2.5 mM extracellular Ca<sup>2+</sup> was rapidly reintroduced (in the continued presence of CPA). The observed [Ca<sup>2+</sup>]<sub>i</sub> response was considered to represent SOCC-mediated Ca<sup>2+</sup> influx.

The observed Ca<sup>2+</sup> influx was characterized using various pharmacologic inhibitors of other Ca<sup>2+</sup> influx mechanisms. The entire protocol was performed in the presence of one of the following agents: (1) 1 nM  $\omega$ -conotoxin MVIIA to inhibit N-type Ca<sup>2+</sup> channels; (2) 1 nM  $\omega$ -agatoxin IVA to inhibit P/Q-type Ca<sup>2+</sup> channels; (3) 5  $\mu$ M mibefradil (Sigma, St. Louis, MO) to inhibit T-type Ca<sup>2+</sup> channels; (4) 10  $\mu$ M KBR to inhibit NCX; (5) 10  $\mu$ M SKF-96365; or (6) 1  $\mu$ M or 1 mM Ni<sup>2+</sup> or La<sup>3+</sup>. To eliminate the contribution of voltage-gated channels *per se*, an additional set of experiments was performed in the simultaneous presence of nifedipine, conotoxin, agatoxin, and mibefradil.

Because Ca<sup>2+</sup> influx may occur *via* several mechanisms that are not dependent on store depletion, a set of control experiments was performed where, after removal of extracellular Ca<sup>2+</sup>, ER stores were not depleted (no CPA exposure). Extracellular Ca<sup>2+</sup> was then reintroduced as for the SOCC experiments.

### Effect of Volatile Anesthetics on Store-operated $Ca^{2+}$ Influx

Store-operated  $Ca^{2+}$  channel-mediated  $Ca^{2+}$  influx was first established by performing a control CPA protocol as above. Cells were then washed for 15–20 min with Hanks balanced salt solution to replenish ER  $Ca^{2+}$  stores. Extracellular  $Ca^{2+}$  was then removed, and the cells were reexposed to CPA. When a  $[Ca^{2+}]_i$  response to CPA was observed, the cells were exposed for 1 min to 0.5, 1, or 1.5 MAC halothane, isoflurane, or sevoflurane in the continued presence of CPA. This technique was used to ensure that ER  $Ca^{2+}$  release was itself not influenced by volatile anesthetics, but the anesthetics were present in sufficient concentration before SOCC activation. In the continued presence of CPA and anesthetic, extracellular  $Ca^{2+}$  was rapidly reintroduced. In control experiments, the SOCC protocol was performed twice without anesthetic.

### Statistical Analysis

At least 15 cells were analyzed for each protocol. Data were compared using a paired *t* test because the protocols involved a control followed by anesthetic/drug effects in the same cell. Repeated-measures analysis of variance with drug/anesthetic and anesthetic concentration as variables was used for multiple comparisons, with Bonferroni corrections, and Tukey *post hoc* analyses. A *P* value less than 0.05 was considered significant (two tailed). All data are expressed as mean  $\pm$  SE.

## Results

### Influx-mode NCX

Baseline  $[Ca^{2+}]_i$  concentrations in differentiated PC12 cells ranged between 75 and 110 nM ( $n = 376$ ).  $Na^+$ -loading of cells with 0  $Ca^{2+}$ , 140 mM  $Na^+$  Tyrode's solution resulted in a moderate decrease in  $[Ca^{2+}]_i$  (fig. 1A), presumably due to active efflux-mode NCX and  $Ca^{2+}$  extrusion across the plasma membrane. After this initial decrease,  $[Ca^{2+}]_i$  concentrations stabilized. Subsequent, rapid reintroduction of extracellular  $Ca^{2+}$  and removal of  $Na^+$  resulted in a rapid rate of increase in  $[Ca^{2+}]_i$ . The  $Ca^{2+}$  influx rate (calculated from the steepest portion of the ascending curve) ranged from 3.5 to 14.6 nM/s during the first run of the protocol. Repetition of the protocol in control experiments resulted in a  $5 \pm 3\%$  decrease in influx rate. Accordingly, time-related bias in the protocol was ignored.

The observed influx was significantly inhibited by the NCX inhibitor KBR ( $P < 0.05$ ; fig. 1B;  $n = 22$ ). In contrast to approximately 90% inhibition by KBR, inhibitors of voltage-gated  $Ca^{2+}$  channels had minimal effects, with inhibition ranging from 5 to 9% (fig. 1B;  $n = 18$  for each inhibitor). In the experiment where several inhibitors of voltage-gated channels were present, the total

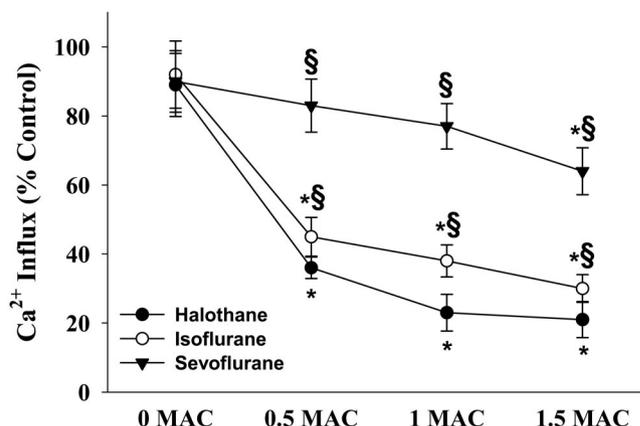


Fig. 2. Effect of halothane, isoflurane, and sevoflurane on  $Na^+$ - $Ca^{2+}$  exchange-mediated  $Ca^{2+}$  influx. Both halothane and isoflurane produced decreases in  $Ca^{2+}$  influx at different concentrations, whereas only greater than 1 minimum alveolar concentration (MAC) sevoflurane produced significant inhibition. \* Significant difference from control ( $P < 0.05$ ). § Significant difference from halothane. Values are presented as mean  $\pm$  SE.

inhibition of influx was 16% ( $n = 30$ ). These data verified that the observed  $Ca^{2+}$  influx occurred *via* NCX.

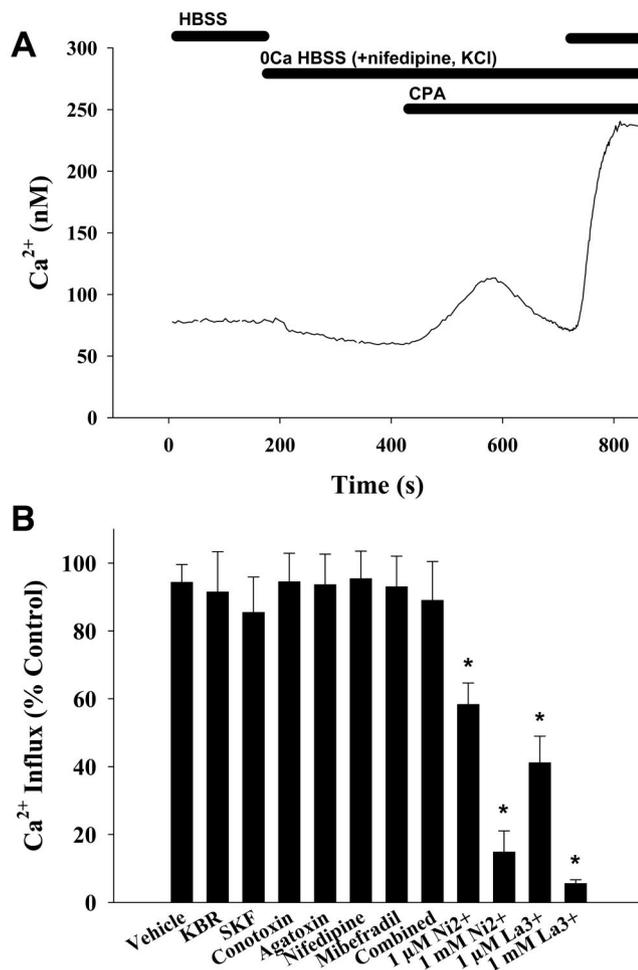
### Effects of Volatile Anesthetics on Influx-mode NCX

Compared with NCX-mediated  $Ca^{2+}$  influx in the initial control part of the protocol, exposure to 0.5, 1, or 1.5 MAC halothane or isoflurane significantly slowed the rate of increase in  $[Ca^{2+}]_i$  concentrations (fig. 2;  $P < 0.05$  compared with control for each halothane or isoflurane concentration;  $n = 26$  for each anesthetic and concentration). For halothane, there was concentration-dependent decrease in NCX-mediated  $Ca^{2+}$  influx. At each concentration, halothane was more potent than isoflurane in inhibiting  $Ca^{2+}$  influx. In comparison to these anesthetics, only sevoflurane at greater than 1 MAC inhibited NCX to a significant extent (fig. 2).

### SOCC-mediated $Ca^{2+}$ Influx

In the absence of extracellular  $Ca^{2+}$ , 1  $\mu$ M CPA resulted in a slow and occasionally transient increase of  $[Ca^{2+}]_i$ , which reached a peak value between 125 and 200 nM ( $n = 467$ ). Subsequent rapid reintroduction of extracellular  $Ca^{2+}$  resulted in a sustained increase of  $[Ca^{2+}]_i$ , which was approximately 100–150% of the peak CPA response (fig. 3).

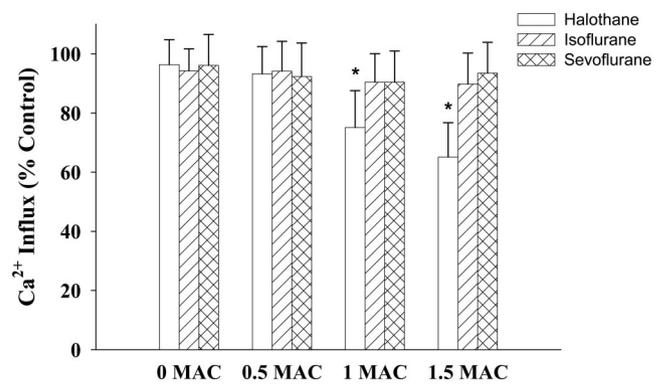
In a previous study, we reported that SOCC-mediated  $Ca^{2+}$  influx is not mediated *via* voltage-gated  $Ca^{2+}$  channels and is inhibited by  $Ni^{2+}$  and  $La^{3+}$ .<sup>39</sup> After control activation of SOCCs with CPA and a wash, cells were exposed to nifedipine,  $\omega$ -conotoxin, agatoxin, mibefradil, 1  $\mu$ M or 1 mM  $NiCl_2$  or  $LaCl_3$  before reactivation of SOCC-mediated  $Ca^{2+}$  influx ( $n = 19$  for each drug). None of the inhibitors of voltage-gated  $Ca^{2+}$  channels resulted in greater than 8% inhibition of the observed influx. In the experiment where several inhibitors of



**Fig. 3.** (A) Schematic of the protocol for examining  $\text{Ca}^{2+}$  influx *via* store-operated  $\text{Ca}^{2+}$  channels in pheochromocytoma (PC12) cells. After initial wash in Hanks balanced salt solution (HBSS), extracellular  $\text{Ca}^{2+}$  was removed, membrane potential was clamped with KCl, and  $\text{Ca}^{2+}$  influx was further prevented with nifedipine. Passive endoplasmic reticulum  $\text{Ca}^{2+}$  release was induced by exposure to cyclopiazonic acid (CPA). Store-operated  $\text{Ca}^{2+}$  channel-mediated  $\text{Ca}^{2+}$  influx was then selectively activated by rapid reintroduction of extracellular  $\text{Ca}^{2+}$ . (B) Characterization of store-operated  $\text{Ca}^{2+}$  channel-mediated  $\text{Ca}^{2+}$  influx. Such influx was not sensitive to any of the inhibitors of voltage-gated  $\text{Ca}^{2+}$  channels, the NCX inhibitor KB-R7943 (KBR), or the receptor-operated  $\text{Ca}^{2+}$  influx inhibitor SKF-96365 (SKF). However, both  $\text{Ni}^{2+}$  and  $\text{La}^{3+}$  significantly inhibited influx. \* Significant difference from control ( $P < 0.05$ ). Values are presented as mean  $\pm$  SE.

voltage-gated channels were present, the total inhibition of influx was 11% ( $n = 22$ ). Both  $1 \mu\text{M}$   $\text{Ni}^{2+}$  and  $\text{La}^{3+}$  significantly inhibited influx (fig. 3;  $P < 0.05$  compared with control), indicating that SOCC-mediated  $\text{Ca}^{2+}$  influx in PC12 cells is sensitive to both ions. In other cells, neither SKF-96365 ( $n = 14$ ) nor KB-R7943 ( $n = 18$ ) significantly inhibited influx (fig. 3).

In control experiments where ER stores were not depleted, reintroduction of extracellular  $\text{Ca}^{2+}$  resulted in a significantly slower rate of  $\text{Ca}^{2+}$  influx compared to that after ER depletion ( $14.4 \pm 4.5\%$  rate of  $\text{Ca}^{2+}$  in-



**Fig. 4.** Effect of halothane, isoflurane, and sevoflurane on  $\text{Ca}^{2+}$  influx *via* store-operated  $\text{Ca}^{2+}$  channels. In contrast to  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchange, only halothane had any significant impact on store-operated  $\text{Ca}^{2+}$  channel-mediated influx. \* Significant difference from control ( $P < 0.05$ ). Values are presented as mean  $\pm$  SE. MAC = minimum alveolar concentration.

crease compared with SOCCs). This influx was significantly inhibited by combined preexposure to conotoxin, agatoxin, nifedipine, and mibefradil ( $91.5 \pm 8.5\%$  reduction compared with control;  $P < 0.05$ ;  $n = 24$ ).

#### Effect of Volatile Anesthetics on SOCC-mediated $\text{Ca}^{2+}$ Influx

Repetition of the SOCC protocol resulted in a 5–8% decrease in the observed  $\text{Ca}^{2+}$  influx (rundown control). In contrast to the significant effects of halothane, isoflurane, and sevoflurane on NCX-mediated  $\text{Ca}^{2+}$  influx, of the three anesthetics, only halothane had any significant inhibitory effect on SOCC-mediated  $\text{Ca}^{2+}$  influx (fig. 4;  $P < 0.05$  compared with control for each anesthetic at each MAC;  $n = 29$  for each anesthetic and concentration).

## Discussion

In the current study, we examined the effects of clinically relevant concentrations of halothane, isoflurane, and sevoflurane on influx-mode NCX and SOCC-mediated  $\text{Ca}^{2+}$  influx in differentiated PC12 cells. We found that halothane, isoflurane, and, to a lesser extent, sevoflurane inhibit NCX-mediated  $\text{Ca}^{2+}$  influx, thus decreasing  $[\text{Ca}^{2+}]_i$ . We also demonstrated the existence of a robust  $\text{Ca}^{2+}$  influx mechanism that is triggered by depletion of ER  $\text{Ca}^{2+}$  stores. Such influx is not mediated by voltage-gated channels. However, in contrast to these inhibitory effects on NCX, volatile anesthetics seem to have only minimal effects on SOCC-mediated  $\text{Ca}^{2+}$  influx in these cells. These data suggest that anesthetic inhibition of neuronal activity may be mediated, at least in part, by inhibition of NCX, but not SOCCs, indicating differential anesthetic sensitivity of  $\text{Ca}^{2+}$  regulatory mechanisms in neuronal cells.

### Methodologic Issues

The current study used differentiated rat pheochromocytoma (PC12) cells as an immortalized model of neuronal cells. These cells have been used extensively for several years, are known to maintain a differentiated neuroendocrine phenotype, and serve as a convenient model system for cell biologic studies on the action of drugs or other interventions on neuronal morphology and function. Although these cells are actually part of the sympathetic nervous system, it is important to consider that they may not necessarily represent "higher-order" neurons (as in the brain and spinal cord), which are in the postdifferentiated state. However, PC12 cells express several of the neuronal receptors, channels, and signal transduction pathways found in higher-order neurons<sup>40</sup> and also release neurotransmitters as do other neurons. Furthermore, PC12 cells respond to neurotrophic factors that modulate higher-order neuronal growth and function, an example being nerve growth factor used for differentiation. Accordingly, the findings of the current study on  $\text{Ca}^{2+}$  regulation using PC12 cells can likely be translated at least to volatile anesthetic effects on  $\text{Ca}^{2+}$  regulation in the sympathetic nervous system and perhaps to cortical and other neuronal cell types that are involved in the clinical manifestations of anesthetics. Whether anesthetic effects on neurotransmission and other neuronal processes in higher-order neurons can also be modeled by PC12 cells remains to be determined.

In the NCX protocol, we observed a small but consistent decrease in  $[\text{Ca}^{2+}]_i$  during  $\text{Na}^+$ -loading of PC12 cells (fig. 1A). Furthermore, in the SOCC protocol, removal of extracellular  $\text{Ca}^{2+}$  also resulted in a small decrease in  $[\text{Ca}^{2+}]_i$ . In previous studies using similar protocols to study NCX (cardiac muscle)<sup>33,41</sup> and SOCCs (airway smooth muscle),<sup>37,39</sup> such a decrease was less obvious and was only occasionally observed. Both of these decreases are most likely due to a robust NCX in PC12 cells. In the NCX protocol, extracellular  $\text{Na}^+$  in the absence of  $\text{Ca}^{2+}$  allows for efflux mode NCX and thus  $\text{Ca}^{2+}$  extrusion and decreased  $[\text{Ca}^{2+}]_i$ , whereas in the SOCC protocol, zero extracellular  $\text{Ca}^{2+}$  again allows for efflux *via* NCX and other mechanisms. Regardless of the mechanisms involved, the decrease in  $[\text{Ca}^{2+}]_i$  is unlikely to have affected the overall results, because in the NCX protocol, the rate of increase of  $[\text{Ca}^{2+}]_i$  (and not absolute concentrations) was measured, whereas in the SOCC protocol, subsequent exposure to CPA increased  $[\text{Ca}^{2+}]_i$ .

### Role of NCX in Neuronal Cells

$\text{Ca}^{2+}$  influx in neuronal cells is known to occur through several mechanisms, including voltage-sensitive  $\text{Ca}^{2+}$  channels, nonspecific cation channels, receptor-operated specific and nonspecific channels, and NCX.<sup>6,14,15</sup> NCX is expressed in high concentrations in

neurons,<sup>16-18,42-44</sup> especially in regions such as synapses, where large amounts of  $\text{Ca}^{2+}$  cross the plasma membrane.<sup>45</sup> In this regard, efflux-mode NCX may contribute to the maintenance of neuronal  $\text{Ca}^{2+}$  homeostasis, especially during depolarization and neurotransmitter release. The role of influx-mode NCX is less clear in neurons that are activated at rapid rates, because depolarization-evoked  $\text{Ca}^{2+}$  transients are extremely brief. However, NCX may regulate resting  $[\text{Ca}^{2+}]_i$  and  $\text{Ca}^{2+}$  store content, thus indirectly modulating transmitter release.<sup>44,46</sup> Repeated depolarizations may increase intracellular  $\text{Na}^+$ , triggering influx-mode NCX, and thus modulating  $[\text{Ca}^{2+}]_i$  concentrations.

Previous studies had suggested the existence of NCX in PC12 cells based on radioactive Na and Ca fluxes.<sup>47</sup> Although adrenal chromaffin cells are known to express NCX,<sup>48</sup> there is relatively little data on NCX in PC12 cells. Our data confirm a very robust NCX activity in these cells. Given the fact that NCX exists in other neuronal cell types, our data are significant because they relate to volatile anesthetic effects on this  $\text{Ca}^{2+}$  regulatory mechanism.

### Volatile Anesthetic Effects on NCX

Given the concurrent effects of anesthetics on multiple  $[\text{Ca}^{2+}]_i$  regulatory mechanisms, particularly  $\text{Ca}^{2+}$  influx channels and ER, as well as the lack of specific inhibitors of the influx and efflux modes of NCX, detailed studies on anesthetic and NCX interactions have been lacking. In fact, most of the published literature, including from our group, exists in cardiac muscle. Hawthorth and Goknur<sup>49</sup> found that halothane, isoflurane, and enflurane all completely inhibit cardiac NCX. In recent studies on adult *versus* neonatal cardiac muscle, we also found that both halothane and sevoflurane inhibit NCX-mediated  $\text{Ca}^{2+}$  influx as well as efflux.<sup>33,41</sup> Furthermore, we were the first to report that both halothane and sevoflurane blunt the relation between NCX-mediated  $\text{Ca}^{2+}$  influx and intracellular  $\text{Na}^+$ , as well as between NCX-mediated  $\text{Ca}^{2+}$  efflux and  $[\text{Ca}^{2+}]_i$  and extracellular  $\text{Na}^+$ .<sup>33</sup>

There are currently no published studies on volatile anesthetic interactions with NCX in neuronal cells. Therefore, the current study is the first to demonstrate an inhibitory effect of clinically relevant concentrations of volatile anesthetics on NCX in neuronal cells. Such an inhibitory effect is consistent with previous findings in cardiac muscle, where NCX-mediated  $\text{Ca}^{2+}$  influx may play a part in overall modulation of  $[\text{Ca}^{2+}]_i$  concentrations,<sup>41</sup> as in neuronal cells. In fact, we found that volatile anesthetics have a proportionately greater inhibitory effect on influx-mode NCX compared with efflux-mode NCX.<sup>33</sup> The results of the current study are interesting in that both halothane and isoflurane seem to potentially inhibit NCX, whereas sevoflurane at less than 1 MAC does not have a significant effect. These data suggest that

volatile anesthetic agents may differ in the mechanisms by which they inhibit neuronal activity in producing anesthesia.

#### *Role of SOCC-mediated Influx in Neuronal Cells*

In nonneuronal cells, there is now considerable evidence, including our own, for  $\text{Ca}^{2+}$  influx *via* SOCCs in response to ER  $\text{Ca}^{2+}$  depletion.<sup>21,37,39</sup> SOCC-mediated  $\text{Ca}^{2+}$  influx (also termed *capacitative  $\text{Ca}^{2+}$  influx*) is thought to serve the purpose of ER  $\text{Ca}^{2+}$  replenishment. However, the transfer of  $\text{Ca}^{2+}$  from the extracellular environs to the ER actually involves an increase in cytosolic  $\text{Ca}^{2+}$ , *i.e.*,  $[\text{Ca}^{2+}]_i$ . Such  $\text{Ca}^{2+}$  influx is thought to occur *via* specific channels rather than receptor-operated or voltage-operated  $\text{Ca}^{2+}$  channels, determined from electrophysiologic data and the sensitivity or insensitivity of these channels to various pharmacologic blockers.<sup>21</sup> ER-mediated  $\text{Ca}^{2+}$  signaling is obviously important in neuronal cells. However, there are little data on SOCC-mediated  $\text{Ca}^{2+}$  influx in this tissue. In the central nervous system, SOCC-mediated  $\text{Ca}^{2+}$  influx has been demonstrated in astrocytes<sup>26</sup> and neuroblastoma cell lines.<sup>27</sup> There is now considerable evidence for SOCC-mediated  $\text{Ca}^{2+}$  influx in PC12 cells.<sup>22,28,29</sup> The results of the current study are consistent with previous findings of a robust SOCC-mediated  $\text{Ca}^{2+}$  entry upon ER depletion.

Initial studies on SOCC-mediated  $\text{Ca}^{2+}$  influx suggested that such influx occurs largely in response to depletion of  $\text{IP}_3$ -sensitive  $\text{Ca}^{2+}$  stores.<sup>21</sup> However, in recent studies using airway smooth muscle,<sup>39</sup> we demonstrated that SOCC activation is dependent not only on  $\text{IP}_3$ -sensitive  $\text{Ca}^{2+}$  stores, but also on ryanodine-sensitive stores (*i.e.*,  $\text{Ca}^{2+}$  release *via* RyR channels). Similar dependence of SOCCs on RyR channels has also been reported in mouse anococcygeus muscle.<sup>50</sup> In the current study, we did not specifically examine  $\text{IP}_3$ -*versus* RyR-dependent SOCCs. However, SOCC-mediated  $\text{Ca}^{2+}$  influx due to release from both stores has been previously demonstrated in PC12 cells.<sup>28</sup> This raises the issue of depletion of both stores using pharmacologic approaches. Several studies have used either CPA or the irreversible sarcoendoplasmic reticulum  $\text{Ca}^{2+}$  adenosine triphosphatase inhibitor thapsigargin. In the current study, the use of the reversible drug CPA allowed for comparison of drug effects in the same cell. Unlike several  $\text{Ca}^{2+}$  channels usually expressed by neuronal cells, SOCCs are not activated by changes in membrane potential.<sup>21</sup> In accordance, in the current study, the robust  $\text{Ca}^{2+}$  influx after ER  $\text{Ca}^{2+}$  depletion by CPA was found to be largely insensitive to nifedipine,  $\omega$ -conotoxin, agatoxin, or mibefradil, confirming that L-type, N-type, P/Q-type, or T-type  $\text{Ca}^{2+}$  channels do not mediate the observed influx. Consistent with other studies in nonneuronal cells, we also found that SOCCs are blocked by  $\text{Ni}^{2+}$  and  $\text{La}^{3+}$ .<sup>39,50,51</sup> Although both ions can

nonspecifically inhibit  $\text{Ca}^{2+}$  influx, inhibition of influx after ER depletion by micromolar concentrations of either ion strongly indicates the presence of SOCCs.

It is interesting that, given the myriad of  $\text{Ca}^{2+}$  channels expressed in neuronal cells, SOCC-mediated influx is still so robust. However, a consistent finding (including in the current study) has been the relatively small increase in  $[\text{Ca}^{2+}]_i$  with inhibitors of the sarcoendoplasmic reticulum  $\text{Ca}^{2+}$  adenosine triphosphatase (*e.g.*, thapsigargin, CPA), suggesting only a small ER store component.<sup>52</sup> SOCC-mediated influx may then functionally compensate for the small ER response with agonist or electrical stimulation. Recent evidence of SOCC-mediated  $\text{Ca}^{2+}$  influx in hippocampal pyramidal and dentate granule cells shows that  $[\text{Ca}^{2+}]_i$  response from such influx is comparable to that induced by *N*-methyl-D-aspartate.<sup>53</sup> Furthermore, such influx seems to be activated by *N*-methyl-D-aspartate. These data suggest that SOCC-mediated  $\text{Ca}^{2+}$  influx plays a role in synaptic plasticity of neuronal cells. Again, given the recent evidence for such influx in other neuronal cells, our data in PC12 cells are of significance, especially because they pertain to volatile anesthetic effects.

#### *Volatile Anesthetics Effects on SOCC-mediated $\text{Ca}^{2+}$ Influx*

As with NCX, there are currently few data on anesthetic effects on SOCC-mediated  $\text{Ca}^{2+}$  influx. In endothelial cells, Tas *et al.*<sup>54</sup> found that isoflurane inhibits histamine-induced  $\text{Ca}^{2+}$  influx *via* SOCCs. However, in another study by the same group, enflurane was found to enhance  $\text{Ca}^{2+}$  influx in rat glioma cells.<sup>55</sup> We recently reported that halothane, isoflurane, and, to a lesser extent, sevoflurane inhibit SOCCs in airway smooth muscle.<sup>37</sup>

In contrast to the limited previous data, the current study found that even high concentrations of volatile anesthetic do not significantly inhibit SOCC-mediated  $\text{Ca}^{2+}$  influx in PC12 cells. Although somewhat surprising, given the potent inhibition of this mechanism in airway smooth muscle, these data raise several issues. SOCC-mediated  $\text{Ca}^{2+}$  influx occurs *via* transient receptor potential (TRP) channels.<sup>24</sup> Cell types differ in the relative expression of TRP isoforms (TRPC subfamily). Furthermore, these isoforms also differ in the extent to which they mediate  $\text{Ca}^{2+}$  influx, as well as their sensitivity to various agonists and modulators. Currently, it is not entirely clear whether SOCCs are mediated *via* different isoforms in different tissues. For example, in hippocampal neuronal cells, TRPC1 and TRPC3 isoforms mediate influx *via* SOCCs.<sup>56</sup> TRPC1 and TRPC3 messenger RNAs were endogenously expressed in PC12 cells.<sup>22</sup> Accordingly, it is possible that neuronal cells differ from other cell types in the relative roles of TRPC isoforms in mediating SOCCs. In this regard, these isoforms may further differ in their sensitivity to inhibition by volatile

anesthetics. Further studies are required to examine this issue.

#### *Clinical Significance of Volatile Anesthetic Effects*

Increase of  $[Ca^{2+}]_i$  plays an important role in neurons, both in synaptic transmission and in neuronal function in general. In this regard, both NCX-mediated and SOCC-mediated  $Ca^{2+}$  influx likely play important roles. NCX may regulate resting  $[Ca^{2+}]_i$  and ER  $Ca^{2+}$  stores, thus indirectly modulating transmitter release. Furthermore, NCX may be triggered by repeated depolarizations that increase intracellular  $Na^+$ . Accordingly, anesthetic inhibition of NCX has the potential to prevent ER  $Ca^{2+}$  repletion and modulate synaptic transmission, thus contributing to anesthesia.

In addition to basic  $[Ca^{2+}]_i$  regulation in neurons, NCX may play a role in neuronal excitotoxicity, e.g., due to *N*-methyl-D-aspartate, by promoting  $Ca^{2+}$  influx in reverse mode.<sup>57</sup> Accordingly, volatile anesthetic inhibition of NCX may provide significant cellular protection against excitotoxic injury from increased  $Ca^{2+}$ . Reverse-mode NCX also contributed to increased  $[Ca^{2+}]_i$  during ischemia-reperfusion injury, and inhibition of NCX has been shown to be neuroprotective *in vitro*. NCX inhibition with KBR has been shown to prevent ischemia-reperfusion injury in the cerebral cortex.<sup>58</sup> Similar NCX inhibition by volatile anesthetics may also help to minimize neuronal reperfusion injury.

In contrast to NCX inhibition, the lack of anesthetic effect on SOCCs may highlight the point that volatile anesthetic inhibition of neuronal function may not represent a generalized, nonspecific inhibition of several regulatory mechanisms. The fact that even higher concentrations of potent anesthetics such as halothane did not inhibit SOCCs suggests that at least one  $Ca^{2+}$  regulatory mechanism may remain intact in neurons exposed to anesthetics, allowing for compensation and recovery from anesthetic effects. How such differential effects of anesthetics on NCX *versus* SOCCs affects synaptic transmission remains to be determined.

Finally, an interesting finding has been the relative lack of effect of sevoflurane on NCX and SOCCs. Comparisons were made between anesthetic agents at the same MAC values. Accordingly, the lack of effect of sevoflurane may be related to lower potency of the agent in modulating NCX or SOCC protein function. Further investigations are necessary to determine whether the three anesthetics differ in their mechanism of interaction with  $Ca^{2+}$  regulatory proteins, regardless of concentration.

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