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Original Article

Volatile Anesthetic Depression of Ca^{2+} Entry Into and Glutamate Release from Cultured Cerebellar Granule Neurons

Carl Lynch III, Ning Miao, Kaoru Nagao, and Joseph J. Pancrazio

ABSTRACT

Background: Volatile anesthetics (VAs) are known to have actions on a variety of ligand- and voltage gated ion channels, and thereby inhibit neuronal function. VA effects mediated by actions on voltage-gated Ca channels (VGCCs) were determined by studying their effects on the depolarization-induced rise in intracellular Ca^{2+} transients and the consequent glutamate release in cultured neonatal rat cerebellar granule neurons.

Methods: Using a glutamate dehydrogenase-coupled assay for glutamate release, and fura-2 to measure intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$), neurons at 37℃ were depolarized by a rapid increase in $[K^+]_o$ from 5 to 55 mM. Actions of halothane, isoflurane, enflurane, and sevoflurane were compared with effects of altered $[Mg^{2+}]_o$, and by specific blockade of L-, P/Q- and/or N-type VGCC by nicardipine, ω-agatoxin IVA, and ω-conotoxin-GVIA, respectively. Whole-cell patch-clamp studies in these same neurons of VGCC Ba^{2+} currents were also performed at 22℃.

Results: Clinical VA concentrations dose-dependently depressed both peak $[Ca^{2+}]_i$ and glutamate release by 35-70%. With N- and/or L-type VGCC blockade, VAs caused a further marked decrease in $[Ca^{2+}]_i$ transients. VAs depressed whole cell patch-clamped Ba^{2+} currents in these granule cell neurons by 35-40%.

Conclusions: VAs depress Ca^{2+} entry by inhibiting a variety of VGCCs, and thereby reduce neuronal glutamate release. This action may contribute to the mechanism of anesthesia as well as provide protection during ischemic insults that cause neuronal injury.

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In mediating loss of consciousness and abolishing response to painful stimuli, volatile anesthetics (VAs) appear both to enhance inhibitory neurotransmission mediated by $GABA_A$ receptors (1, 2) and to inhibit excitatory synaptic transmission (3-5). In the latter case, the effects of VAs appear to be associated with decreased release of glutamate, mediated in part by a reduction in Ca^{2+} influx (3-6). In adrenally-derived PC12 cells, VAs inhibited the K^+-depolarization rise in intracellular Ca^{2+} due to influx via L- and N-type voltage-gated Ca channels (VGCCs) (7). At clinical concentrations and physiologic temperatures, VAs cause parallel and dose-dependent reductions in Ca^{2+} transients and glutamate release from isolated cerebral synaptosomes induced depolarization with 30 mM K^+ (8). These decreases are similar to those seen with reductions in external Ca^{2+} and consistent with VA mediated inhibition of Ca^{2+} entry via VGCCs (8). However, inhibition of Na channels also decrease neurotrans-

mitter release have raised the possibility that VAs primarily prevent the Na channel-mediated depolarization that opens VGCCs (9-11). In addition to possibly mediating components of the anesthetic state, VAs may also mediate neuronal protection (12). The bulk of brain energy utilization is related to glutamatergic neuronal activity (13). A presynaptic decrease in Ca^{2+} entry will decrease glutamate release and thereby decrease post-synaptic Ca^{2+} entry via glutamate activation of NMDA (N-methyl-D-aspartate) receptors, decreasing neuronal energy consumption. In addition, the inhibition of neuronal VGCC release may directly decrease the cellular Ca^{2+} overload that activates autolysis or apoptosis (14).

Cerebellar granule (CG) neurons cultured from neonatal rat are a well-established model of uniform cells which can be employed for toxicological and pharmacological investigation of synaptic transmission (15, 16). Both pharmacological and toxicological investigations, as well as molecular cloning, have defined the diversity of Ca channels in vertebrate neurons. Using a series of potent channel inhibitors and toxins, five distinct high voltage activated (HVA) Ca^{2+} currents (L-, P-, Q-, N-, and R-type) have been identified and are present in in rat CG neurons (17). Four distinct ion conducting α Ca channel subunits have been defined by molecular biologic techniques that correspond to these electrophysiological types: $Ca_V1.2$ (L-type, α_C), Ca_V 2.1 (P and Q are splice variants, α_A), Ca_V 2.2 (N-type, α_B), and Ca_V 2.3 (R-type, α_E) (18). We have previously demonstrated the ability of VA's to depress Ca^{2+} currents through all four channels types when expressed in Xenopus oocytes (19). The present study was undertaken to determine to what extent VAs alter $[Ca^{2+}]_i$ transients in CG neurons mediated by these multiple types of VGCCs, and the resulting glutamate release evoked by the Ca^{2+} entry.

METHODS

Cell Isolation and Culture

Following the National Institutes of Health (NIH) Guide for the care and use of laboratory animals and a protocol approved by the University of Virginia Animal Research Committee, CG neurons were prepared by a modification of the method of Novelli et al. (15) using cerebella isolated from 5- to 7-day-old Sprague-Dawley rat pups. The tissue was coarsely chopped, trypsinized (Type III) for 45 minutes at 37℃, followed by addition of DNase I and trypsin inhibitor and gentle centrifugation. The supernatant was discarded, and the pellet was triturated, and after 5 minutes, $MgCl_2$ (2.5 mM) and $CaCl_2$ (0.1 mM) were added to solution. The neuronal suspension was filtered through 70 μm mesh and recentrifuged for 2 minutes. Neurons from the resuspended pellet (2×10^6) were plated onto poly-L-lysine-coated glass coverslips (11 mm × 22 mm) cultured in basal Eagle's medium with 10% fetal calf serum, 2 mM glutamine, 100 μg/ml gentamicin and 25 mM K^+. Glial cell proliferation was prevented by addition of 10 μM cytosine arabinoside 24 hours after plating. Granule neurons were maintained in 5% CO_2 : 95% air at 37℃ and were used 4 to 10 days after isolation. A small series of neurons was grown in solution containing 5 mM KCl. Biochemical reagents, buffers and toxins were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise indicated. Halothane was obtained from Halocarbon Laboratories (Riveredge, NJ), sevoflurane from Abbott Laboratories (North Chicago, IL), isoflurane and enflurane from Anaquest/Ohmeda (Liberty Corner, NJ).

$[Ca^{2+}]_i$ Measurement in Cultured Granule Neurons

For measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), neurons on coverslips were incubated at 37℃ for 20 minutes in basal medium containing 3 μM fura-2-AM, BSA 16 μM and (in mM): NaCl 153, KCl 3.5, $NaHCO_3$ 5, KH_2PO_4 0.4, $MgSO_4$ 1.2, $CaCl_2$ 1.3, glucose 5, N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES) 20 with pH adjusted to 7.4. In some experiments HEPES was substituted for TES with no alteration in behavior. After washing the neurons twice in fura-2-free solution, coverslips were inserted into a holder and placed in a 2 ml cuvette and washed twice more with 2 ml fresh medium. $[Ca^{2+}]_i$ was determined at 37℃ in a PTI (Photon Technology Incorporated, Monmouth Junction, NJ) DeltaScan luminescence spectrofluorometer equipped with a cuvette warmer and magnetic stirrer to ensure adequate mixing during each experiment. Ca^{2+} in-

flux into neurons was initiated with addition of 33 μl of 3.0 M KCl, which increased $[K^+]_o$ from 5 to 55 mM. Fluorescence at 510 nm was determined for alternating excitation wavelengths of 340 and 380 nm with fluorescence (340/380) ratios collected every 0.5 to 1.9 seconds for 60 to 180 seconds. Subsequent calibration was carried out by determining maximum and minimum fluorescence ratios using 10 μM ionomycin for maximum (Ca^{2+}-saturated) values and 10 mM ethylene glycol-bis (-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA) for minimum values for each coverslip. $[Ca^{2+}]_i$ was calculated according to the standard formula using a Ca^{2+}-fura-2 K_d of 224 nM (20). The signal amplitude and time course were extremely reproducible among cells on a given day. Addition of 100 μmoles of 1M NaCl instead of KCl elicited no response, suggesting change in tonicity by itself had minimal effect. In the presence of 1 mM EGTA and in the absence of added Ca^{2+}, extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_o$) was less than 100 nM and KCl addition elicited no $[Ca^{2+}]_i$ transient. To further verify the assay, Mg^{2+} ($[Mg^{2+}]_o$) was adjusted to change the amount of entering Ca^{2+} as well as the resulting glutamate release.

Measurement of Neuronal Glutamate Release

Glutamate release was measured in CG neurons at 4-7 days using a glutamate dehydrogenase (GluDH)-coupled assay (Boehringer Mannhein GmbH, Germany). As described (21, 22), 50 U/ml GluDH was employed in the presence of 1 mM $NADP^+$ to catalyze the formation of α-ketoglutarate and the fluorescent species NADPH from glutamate. NADPH fluorescence was excited at 340 nm and measured at 460 nm using the PTI spectrofluorometer. The coverslip of granule neurons was washed in buffer solution and then incubated at 37℃ for 5 min in a 2 ml cuvette containing (in mM): 145 NaCl, 5 KCl, 1.3 $MgCl_2$, 1.5 $CaCl_2$, 1.2 NaH_2PO_4, 10 glucose, and 20 HEPES, pH 7.4. As in the $[Ca^{2+}]_i$ transient study, glutamate release was activated by adding KCl to achieve a final concentration of 55 mM, while monitoring the change in NADPH fluorescence for 300 seconds at a sampling rate of 1-2 Hz. The fluorescence signal in this setting increased to a value 10-20% above the baseline fluorescence, with a typically stable plateau being reached within 10 seconds.

To calibrate the fluorescent response to glutamate release, studies were performed with direct addition of NADPH or glutamate under identical conditions. Addition of NADPH in the cuvette solution to obtain a 0.2, 0.5, and 1.0 μM concentration resulted in abrupt increases in the fluorescence signal of 0.92 ± 0.21, 1.94 ± 0.86, and $3.1 \pm 1.31 \times 10^5$ counts per second (cps), respectively ($\pm$ SD, n=5). When glutamate was added (in aliquots of 0.5 mM solution) to solutions containing 50 mg/ml GluDH enzyme solution, the fluorescence signal increased with an exponential time course time constant of ~60 seconds at 37℃. When the added (glutamate) was 0.2, 0.5 and 1.0 μM (equimolar to the increases in [NADPH]), the respective steady-state increases in fluorescence signals were 0.89 ± 0.19, 1.83 ± 0.29, and $2.89 \pm 0.30 \times 10^5$ cps, or ~95 percent of the NADPH values. The close agreement of the fluorescence signal between the same quantity of NADPH and glutamate suggests that the glutamate reaction producing NADPH proceeded to completion. In control experiments, when [glutamate] was abruptly increased to 0.2, 0.5 and 1.0 μM in the NADPH buffer and GDH mixture in the presence of halothane or isoflurane, and no difference was observed in the rate or extent of fluorescence increase seen in their absence.

Upon addition of KCl and depolarization of the neurons, there was a sudden increase in the NADPH fluorescence signal, followed by a much smaller and slower increase, which typically stabilized by 5 to 15 seconds (Figure 1b, 2b, 3b). The increase in the fluorescence signal was typically on the order of $0.4\text{-}1.5 \times 10^5$ cps, reflecting a final metabolism of 0.2-0.4 nmoles of released glutamate. The maximum value varied with the degree of confluence and coverage of neurons on the coverslips. Compared to the addition of glutamate in solution, the stabilization of the fluorescence signal in the presence of depolarization-induced glutamate released from neurons was far more rapid. Such rapidity suggests that there must be rapid release of a high concentration of glutamate (>40 nmoles yielding >20 μM), followed by rapid arrest (≤1 second) of glutamate release, as well as substantial local glutamate uptake into neurons which

would then cause cessation of NADPH production in the first few seconds. A high concentration of glutamate (>1 mM) with rapid reuptake has been predicted to be found in synaptic clefts (23), while a high-capacity system for glutamate uptake in neurons (24) could account for the rapid stabilization of the signal.

In additional previously reported control experiments, $GABA_A$ receptors (chloride ion channels) were blocked using 100 μM bicuculline, $GABA_B$ receptors were activated by 10 μM baclofen, NMDA glutamate receptors were inhibited by D-(−)-2-amino-5-phosphonovaleric acid (AP-5), or intracellular Ca^{2+} was mobilized by 5 mM caffeine. None of these separate interventions had any significant action on the depolarization-evoked Ca^{2+} transient or glutamate release (25). In that same study, Na channel blockade by 10 μM tetrodotoxin caused an 11% decrease in the Ca^{2+} transient peak (a non-significant decrease in glutamate release), while intracellular Ca^{2+} immobilization with 10 μM ryanodine caused a 14% decrease in glutamate release (no effect on the Ca^{2+} transient).

Anesthetic and Drug Administration

Prior to either type of experimental study, CG neurons were incubated in the cuvette for 5 minutes in VA-equilibrated solution, which was generated by bubbling filtered VA-containing air which had passed through anesthetic vaporizers (Ohmeda, Madison, WI) calibrated to deliver the specified percent vapor in air. VA vapor concentrations were approximately 0.8 and 1.6 times the minimal alveolar concentration (MAC) value for rats (that concentration at which 50% of rats do not respond to painful stimulation) (26). As periodically verified by gas chromatography, 0.75 and 1.5% halothane yielded aqueous concentrations of 0.25 and 0.5 mM; 1.3 and 2.5% isoflurane yielded 0.23 and 0.42 mM; 2% and 4% sevoflurane yielded 0.22 and 0.43 mM; 1.7 and 3.5% enflurane yielded 0.35 and 0.70 mM enflurane. Solutions were sampled at 37℃ and aqueous concentrations typically varied by ±10%. VA-containing air continually flowed through the cuvette head-space to prevent VA loss to the atmosphere. Control solutions were bubbled with filtered air only. A five minutes incubation was sufficient to achieve a stable effect of the anesthetics, nicardipine or ω-agatoxin-IVA (Aga-IVA; Alexis Biochemical, San Diego, CA); a 20 minutes prior exposure to ω-conotoxin-GVIA (Ctx-GVIA) was found to be necessary to achieve its maximum effect on either $[Ca^{2+}]_i$ or glutamate release.

Whole-Cell Patch-Clamp Studies

For electrophysiological studies, neurons grown on cover slips under conditions identical to those for the spectrophotometric studies were placed at the bottom of a recording chamber mounted on an inverted microscope where bathing solutions could be exchanged. Prior to establishing the whole-cell-recording configuration, the external bathing solution contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, adjusted to pH 7.4 with 1 N NaOH. The patch pipette solution contained (in mM): 108 $CsMeSO_4$, 10 CsCl, 9 EGTA, 24 HEPES, 4 Mg-ATP, 0.3 GTP, adjusted to pH 7.3 with 1 N CsOH. Once whole-cell recording was achieved, the bathing solution was replaced with one that would eliminate potentially interfering K and Na currents (in mM): 160 TEA-Cl, 5 $BaCl_2$, 10 HEPES, pH 7.3 with 1 N CsOH.

Standard whole-cell voltage-clamp methods were employed using the Axopatch 200 patch clamp amplifier (Axon Instruments, Foster City, CA). Data acquisition was performed using a pClamp system version 5.5.1 (Axon Instruments) coupled with an IBM-compatible, 386-based microcomputer. Patch electrodes were prepared from borosilicate glass 1B150F-3 (World Precision Instruments), heat polished, and had a resistance less than 5 MΩ when filled with internal solution. All experiments were conducted at room temperature (20-22℃). Four to six minutes after initiating whole-cell recording configuration, neurons were typically voltage-clamped at -80 mV to establish a stable baseline and maximize currents by reducing steady state inactivation. To define the current-voltage relation, I_{Ba} was triggered by step depolarizations 70 msec in duration from -40 to +40 mV. After control measurements the preparation was superfused for 4 to 6 minutes with solution pre-equilibrated with either halothane or isoflurane (produced by bubbling the solution at room temperature) and measurements repeated. Anesthet-

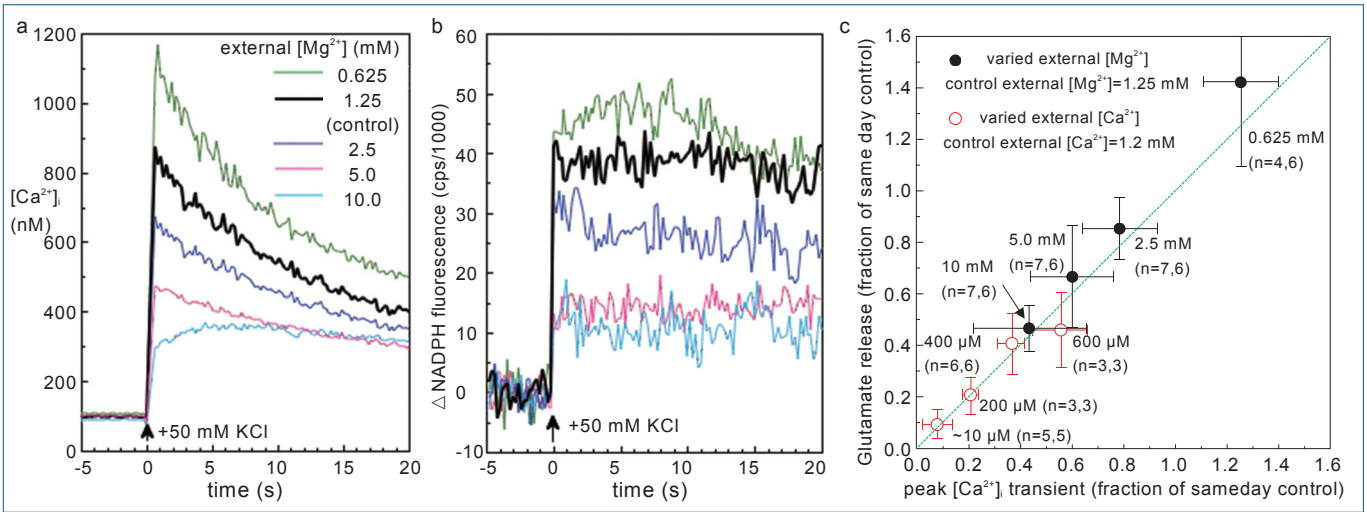

Figure 1. Effects of Altered $[Mg^{2+}]_o$ or $[Ca^{2+}]_o$ on the $[Ca^{2+}]_i$ Transients and Glutamate Release from Cultured Neonatal Rat Granule Cell Neurons Induced by the Sudden Depolarization by Increasing $[K^+]_o$ from 5 to 55 mM.
a, The differing $[Ca^{2+}]_i$ transient observed; decreased $[Mg^{2+}]_o$ increased the $[Ca^{2+}]_i$ transient while increasing $[Mg^{2+}]_o$ decreased the response. b, Changes in NADPH fluorescence which reflect the release of glutamate from cultured neurons (see legend in a). Decreased $[Mg^{2+}]_o$ enhanced glutamate release while increases in $[Mg^{2+}]_o$ decreased the glutamate release. c, Relationship between fractional changes in peak $[Ca^{2+}]_i$ and glutamate release with varied $[Mg^{2+}]_o$ (●) or $[Ca^{2+}]_o$ (○) used from reference (25). $[Mg^{2+}]_o$ or $[Ca^{2+}]_o$ is indicated beside each point, which represents the mean ± SEM for n $[Ca^{2+}]_i$ and glutamate release experiments, respectively. For each experiment, the result was expressed as the fraction of the control measurement ($[Mg^{2+}]_o$ = 1.25 mM, $[Ca^{2+}]_o$ = 1.2 mM) performed the same day. The line represents the line of unity relation between glutamate release and the peak $[Ca^{2+}]_i$ transient. The correlation coefficients (R^2) for relation between glutamate release and the peak $[Ca^{2+}]_i$ was 0.999 for altered $[Mg^{2+}]_o$ (slope= 1.17) and 0.939 for decreased Ca^{2+} (slope=0.81).

ic solution was washed out for 5-8 minutes before recording recovery currents. Standard P/n analysis was used to estimate and subtract leakage and capacitative currents. The higher solubility of the anesthetics at room temperature produced aqueous concentrations approximately 60-70% higher than those at 37℃. In other experiments a depolarization to -10 mV was applied for 400-450 msec. A 9.5 sec depolarization to 0 mV was applied to duplicate the prolonged depolarization obtained with application of 55 mM K^+.

Statistics and Analysis

For neurons at the same day in culture and growing with a similar density, KCl depolarization elicited extremely uniform control responses for either $[Ca^{2+}]_i$ transients or glutamate release, varying by less than 8%. Results of $[Ca^{2+}]_i$ measurements are reported and compared as absolute values and also as fraction of same day control, while glutamate measurements are only reported in the latter format. Unless otherwise indicated, results are expressed as sample mean ± sample standard error (SEM). Results were compared among anesthetics and drugs by ANOVA and the Protected Least Significant Difference (PLSD) Test.

RESULTS

Control Studies

As shown by the control response in Figure 1a, a sudden increase in $[K^+]_o$ to 55 mM caused an increase in $[Ca^{2+}]_i$ from 80-110 nM to over 800 nM, subsequently declining to less than one-half the peak value within 20 seconds, with a stable plateau of 160-210 nM reached at 50-90 seconds (not shown). With 1 mM EGTA added to Ca^{2+}-free solution, $[Ca^{2+}]_o$ was reduced to less than 100 nM; the $[Ca^{2+}]_i$ transient and glutamate release were completely abolished (not shown), verifying the requirement for external Ca^{2+} to elicit glutamate release. To alter Ca^{2+} entry and consequent glutamate release, the $[Mg^{2+}]_o$ was halved (0.625 mM) or increased to 2.5, 5.0 and 10 mM. In response to the sudden KCl depolarization, reduced $[Mg^{2+}]_o$ caused an increase in the

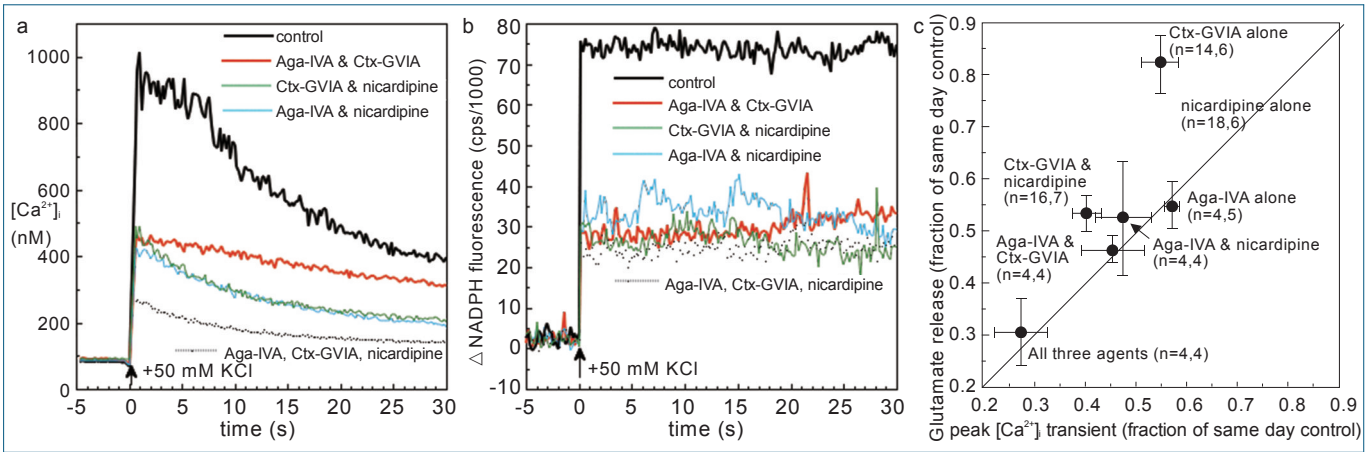

Figure 2. Effects of Combinations of Specific Voltage-gated Ca Channel (VGCC) Blockade upon $[Ca^{2+}]_i$ Transients and Glutamate Release from Cultured Neonatal Rat Granule Cell Neurons.

ω-Conotoxin GVIA (100 nM) was used to block N-type channels, 1 μM nicardipine to block L-type channels, and 100 nM ω-agatoxin-IVA to block P/Q-type channels. Ca^{2+} entry and glutamate release were activated with rapid depolarization by increasing $[K^+]_o$ from 5 to 55 mM. a, $[Ca^{2+}]_i$ transients observed under control conditions and in the presence of differing combinations of VGCC block as indicated. b, Change in NADPH fluorescence reflecting glutamate release for specific VGCC blockade combinations (legend identical to a). c, Relationship between fractional changes in peak $[Ca^{2+}]_i$ and glutamate release with blockade of different individual or combinations of VGCCs. Specific blockers are indicated beside each point, which represents the mean ± SEM for n $[Ca^{2+}]_i$ and glutamate release experiments, respectively. For each experiment, the result was expressed as the fraction of the control measurement performed the same day. The line represents the line of unity relation between glutamate release and the peak $[Ca^{2+}]_i$ transient. The fractional decrease from control was significant ($P<0.05$) for toxin and drug effects on both the Ca^{2+} transient and glutamate release, except for the decrease in glutamate release caused by Ctx-GVIA ($P<0.10$).

peak Ca^{2+} transient and glutamate release (Figure 1) of 25 ± 15 (n=4, P<.05) and 42 ± 33 percent (n=6, P<0.05). Increases in Mg^{2+} decreased Ca^{2+} entry, causing similar proportional declines in peak Ca^{2+} transient and glutamate release. In Figure 1c, the decrease in the glutamate release signal is plotted against the decrease in the peak of the Ca^{2+} transient (peak minus basal) for values normalized to the same day control amplitude. There is clearly a strong linear correlation between glutamate release and peak Ca^{2+} transient.

In addition, using this CG neuron preparation we previously demonstrated that pretreatment with 1 μM ryanodine or 5 mM caffeine did not alter such Ca^{2+} transients nor glutamate release (25), indicating little or no role for Ca^{2+} release from intracellular stores for K^+-evoked $[Ca^{2+}]_i$ transients in these neurons.

The contribution of Ca^{2+} current through L-type, N-type, or P/Q-type VGCC to the $[Ca^{2+}]_i$ transient was determined by use of 1 μM nicardipine, 100 nM Ctx-GVIA, or 100 nM Aga-IVA, respectively (27-29). Each blocker decreased the peak Ca^{2+} transient to about 55-60 percent of control (Figure 2c). While Ctx-GVIA and Aga-IVA caused less depression of the plateau $[Ca^{2+}]_i$ measured at 55 s (74±19 and 66±22 percent control, respectively; P<0.05 paired t-test) than of the peak $[Ca^{2+}]_i$, nicardipine in contrast caused greater depression of plateau $[Ca^{2+}]_i$ (27±13 percent control; P<0.01 paired t-test) than peak. Reductions in glutamate release were more variable (Figure 2c). The more modest effects of Ctx-GVIA in depressing glutamate release compared to Aga-IVA or nicardipine agree with previous reports suggesting that N-type channels contribute modestly to glutamate release in these neurons (22, 30). When two VGCC toxins were combined the $[Ca^{2+}]_i$ transient decreased even more to 40-45% of control (Figure 2a, c). However, the less than additive effect suggests that additive inhibition of the $[Ca^{2+}]_i$ influx may be due in part to overlapping drug sensitivity (22). When 1 μM nicardipine was combined with either Aga-IVA or Ctx-GVIA there was more profound depression of plateau of $[Ca^{2+}]_i$. When two VGCC types were blocked, glutamate release (NADPH signal) showed a fractional depression similar to that observed for peak $[Ca^{2+}]_i$ (Figure 2c). When the three

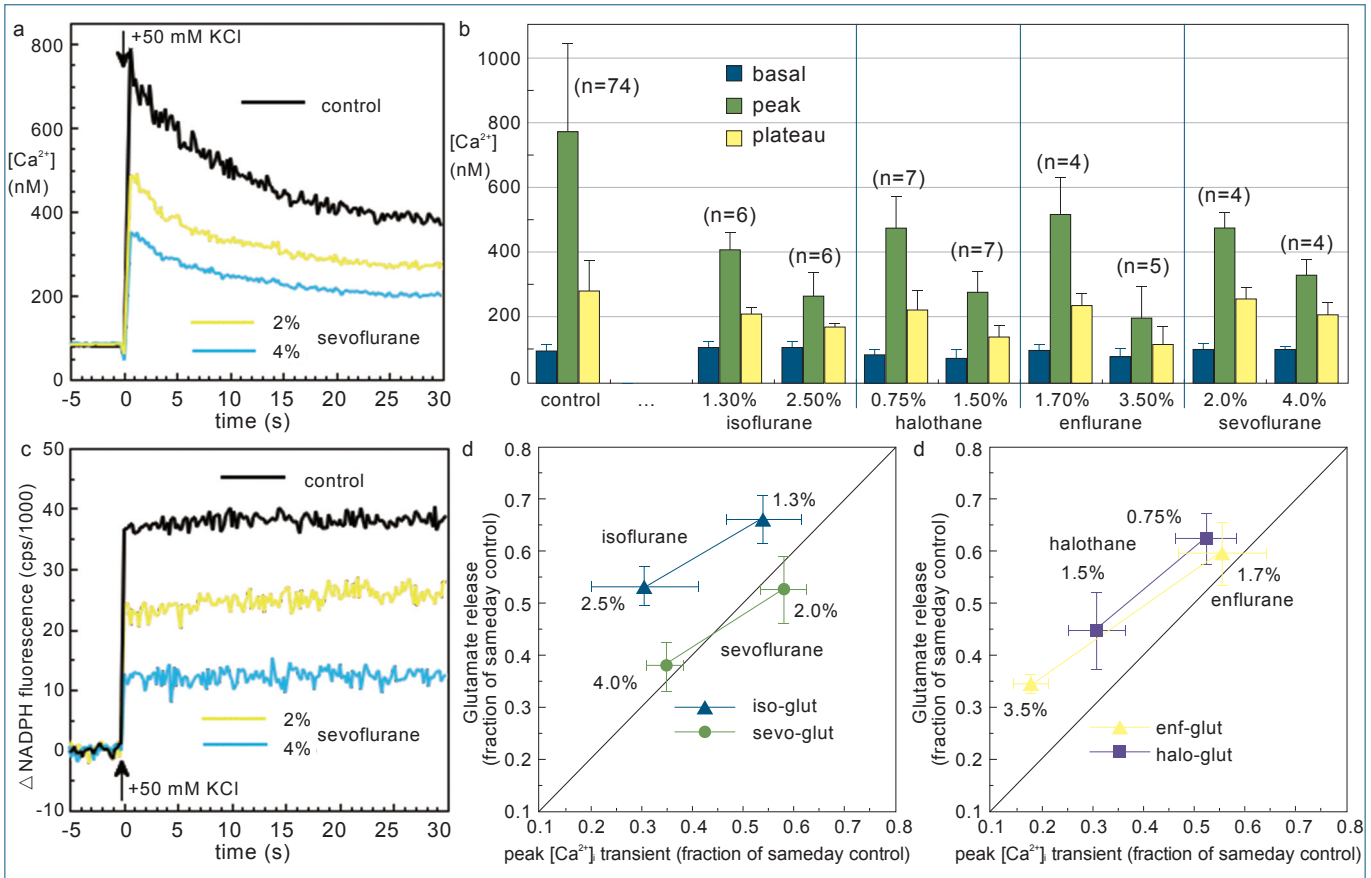

Figure 3. Alteration in the KCl-Depolarization Induced $[Ca^{2+}]_i$ Transient and Glutamate Release Caused by Volatile Anesthetics (VAs) in Cerebellar Granule Neurons.

a, $[Ca^{2+}]_i$ transients for control and in solutions equilibrated with air containing 2 or 4% sevoflurane (0.22 or 0.43 mM). Traces are derived from the calibrated fura-2 340/380 fluorescence ratio. b, Basal (prior to depolarization), peak and plateau (measure at 55-60 seconds) $[Ca^{2+}]_i$ observed in neurons in response to a 50 mM KCl depolarization. Values are mean ± SD for control and in the presence of two equipotent concentrations of isoflurane, halothane, enflurane and sevoflurane. c, Alteration in glutamate release as assessed by the change in NADPH fluorescence for control and in the presence of sevoflurane. d and e, Relationship between the peak $[Ca^{2+}]_i$ and glutamate release for two equipotent concentrations (0.8 and 1.6 times the minimal alveolar concentration (MAC), equivalent to the EC_{50}) of isoflurane, sevoflurane, enflurane and halothane. Anesthetic concentrations in the gas phase (as percent atmosphere are indicated beside each point, which represents the mean ± SEM for n $[Ca^{2+}]_i$ and glutamate release experiments, respectively. For each experiment, the result was expressed as the fraction of the control measurement performed the same day. Line represents line of the unity relation between glutamate release and the peak $[Ca^{2+}]_i$ transient.

agents were applied, the KCl-evoked Ca^{2+} transient and glutamate release were similarly reduced to 28 ± 11 and 30 ± 13 percent of control (n=4), respectively.

Volatile Anesthetic Effects on the $[Ca^{2+}]_i$ and Glutamate Release

VAs did not alter the basal $[Ca^{2+}]_i$, but markedly depressed the K depolarization-induced $[Ca^{2+}]_i$ transient, decreasing both peak and plateau (Figure 3a for 0.22 and 0.43 mM sevoflurane; 2 and 4% gas phase). Average values of the calculated basal, Ca^{2+} transient peak and plateau for control neurons and in the presence of 0.8 and 1.6 times anesthetic MAC (EC_{50}) are shown in Figure 3b. The basal $[Ca^{2+}]_i$ of 80-110 nM increased on depolarization to a peak $[Ca^{2+}]_i$ transient averaging ~770 nM, which typically fell to a plateau of ~ 270 nM, although considerable variation was observed resulting in the broad range (± SD) of $[Ca^{2+}]_i$. Depression of the Ca^{2+} transient caused by sevoflurane was similar to that observed by equivalent 0.8 and 1.6 MAC of the other anesthetics, decreasing both the peak and plateau to 55-70 and 26-37% of control, respectively.

Figure 3c shows the increase in NADPH fluo-

rescence attributable to glutamate release, for control and in the presence of 2 and 4% sevoflurane. The reduction in amplitude of the response is similar to that observed when Ca^{2+} entry was reduced by decreased $[Ca^{2+}]_o$, increased $[Mg^{2+}]_o$, or by specific VGCC blockade. In Figure 3d and e, the VA-induced decrease in the glutamate release signal is plotted against the decrease in the peak of the Ca^{2+} transient. The depressant effect of the equivalent anesthetic concentrations is similar and clearly concentration-dependent in that 1.6 MAC caused approximately twice the depression of that seen with 0.8 MAC of the anesthetic.

To determine if specific VGCC channels were altered by the anesthetics, the decrease in the Ca^{2+} transients were observed when 2.5% isoflurane or 1.5% halothane was combined with L-type VGCC block by 1 μM nicardipine and/or N-type VGCC block by 100 nM Ctx-GVIA. The combination of the depressant effect of 1.5% halothane or 2.5% isoflurane with either L-type or N-type VGCC blockade caused a further decrease of the $[Ca^{2+}]_i$ transient by an additional 20-40% compared to the drug or toxin by itself (Figure 4a-c). Therefore, more than one type VGCC is blocked by the anesthetics. Conversely, since nicardipine or Ctx-GVIA can further augment the depression of the anesthetics, such additional depression also implies that not all L- or N-type channels are completely inhibited by 1.5% halothane or 2.5% isoflurane. In the presence of combined L- and N-type channel block, the additional anesthetic-induced decrease in the Ca^{2+} transient was particularly profound: 1.5% halothane or 2.5% isoflurane further reduced the Ca^{2+} transient mediated by non L-, non-N-type VGCCs (40 percent of control) to only 4-6 percent of control (Figure 4d). Even 0.75% halothane or 1.3% isoflurane resulted in a significant decrease to 18-28 percent of control.

In contrast to neurons grown in 25 mM K^+, the Ca^{2+} transient observed in neurons grown in 5 mM K^+ media was much smaller, increasing from a basal $[Ca^{2+}]_i$ of 49 ± 17 nM to a peak of 175 ± 80 nM, and subsequently decreasing to a plateau of 107 ± 35 nM (n=21). The smaller Ca^{2+} transient was depressed by nicardipine and Ctx-GVIA to 30 and 34 percent of control, respectively, suggesting that L- and N-type Ca channels contributed similarly to Ca^{2+} entry. 1.5% Halothane1.5% depressed the smaller transient peak $[Ca^{2+}]_i$ and plateau to ~46 and ~50 percent of control, respectively (n=3), similar to the inhibition seen in neurons cultured in 25 mM K^+.

Anesthetic Effects on Whole Cell Ca^{2+} Currents

To determine if the depression of $[Ca^{2+}]_i$ transients caused by anesthetics was reflected by a similar effect on voltage gated Ca^{2+} currents, these were examined by the whole cell patch clamp technique. Ba^{2+} was substituted for Ca^{2+} as the charge-carrying ion to enhance the inward current. Under control conditions with -80 mV holding potential, the peak I_{Ba} elicited by a test pulse to -10 or 0 mV averaged -141 ± 91 pA (n=29), with substantial variability in amplitude present among the neurons studied. Peak I_{Ba} was significantly and reversibly diminished by 2.5% isoflurane to 68 ± 8 percent of control ($P<0.001$, n=11, Figure 5a) and by 1.5% halothane to 67 ± 11 percent of control ($P<0.02$, n=5). Anesthetic treatment had no obvious effect on the voltage-dependence of current-voltage relationship (Figure 5b); however, this observation should be interpreted with caution since there are multiple VGCC types in these neurons. Although the amplitude and time course of I_{Ba} varied among the neurons over a 450 ms depolarization, the current decrease seen with application of either 2.5% isoflurane or 1.5% halothane showed little difference from that seen by simply scaling the control response (Figure 5 c, d), suggesting no VA effect on the rate of inactivation of the inward currents.

To determine the relative sensitivity of L- and N-type VGCC to the anesthetics, we exposed CG neurons to the combination of 1 μM nicardipine and 100 nM Ctx-GVIA. Since high concentrations of divalent cations, particularly Ba^{2+}, are known to inhibit N-type Ca channel blockade by Ctx-GVIA (31), neurons were pretreated with Ctx-GVIA for 30 minutes before recording. With the elimination of N-type Ca channels, 2.5% isoflurane inhibited the remaining I_{Ba} (140 ± 41 pA; n=8) by 40 ± 3 percent, slightly more than the 32% depression of I_{Ba} in the absence of the toxin pretreatment ($P<0.05$). In the presence of both 100 nM Ctx-GVIA and 1 μM nicardipine, 2.5% isoflurane decreased the remaining

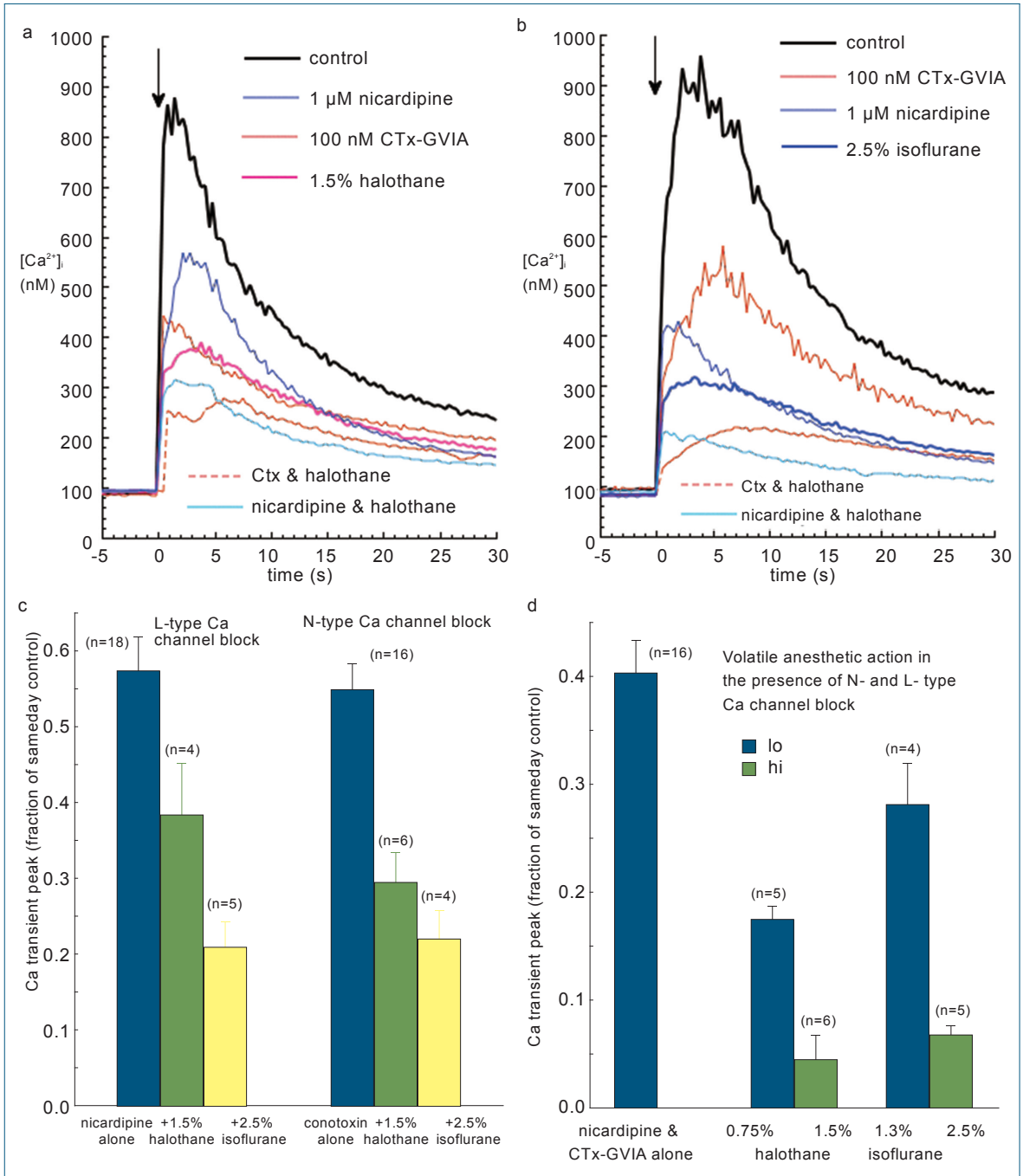

Figure 4. The Effect of Isoflurane or Halothane in the Presence of L-type and or N-type VGCC Blockade. The depression in the $[Ca^{2+}]_i$ transient due to specific L-type Ca channel block with 1 μM nicardipine or N-type Ca channel blockade 100 nM Ctx-GVIA is shown, in the presence and absence of 1.5% halothane (a) or 2.5% isoflurane (b). Ca^{2+} transients in CG neurons were elicited by depolarization with 55 mM KCl as indicated at the arrow. c, Mean depression of the $[Ca^{2+}]_i$ transient peak by either 1 μM nicardipine or 100 nM ω-Ctx-GVIA, in the presence or absence of 1.5% halothane or 2.5% isoflurane. d, Mean depression of the $[Ca^{2+}]_i$ transient peak by both 1 μM nicardipine and 100 nM ω-Ctx-GVIA, in the presence or absence of 0.75% and 1.5% halothane, or 1.3% and 2.5% isoflurane.

peak I_{Ba} from the control value of 143 ± 20 pA by 37 ± 3% (n=5), suggesting that unblocked P/Q and R-type current were inhibited by VAs to a similar degree as the N- and L-type channels.

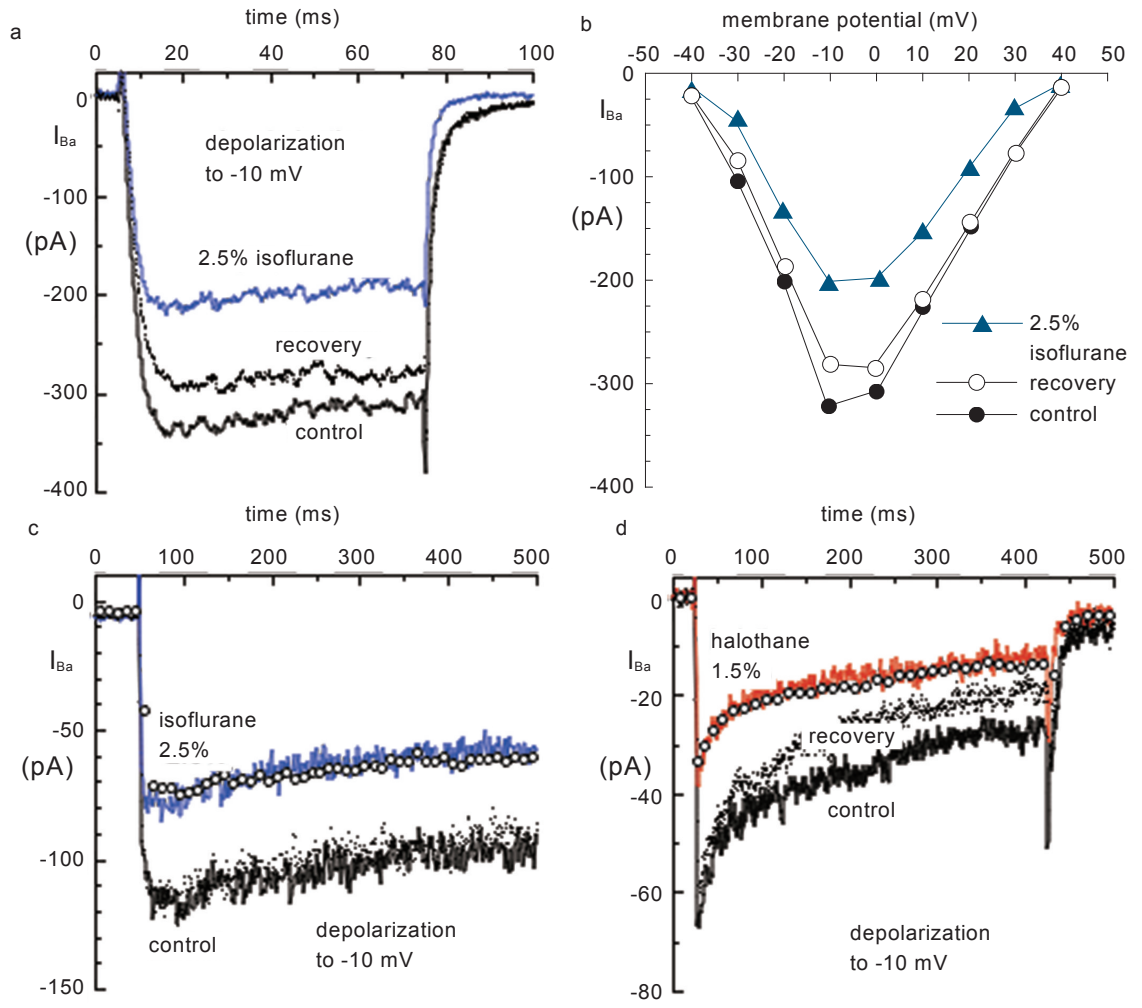

Figure 5. Anesthetic Actions on Ca Channel Currents in Cultured Rat CG Neurons at 21℃.
Neurons at 4-8 days in culture were whole cell patch clamped with pipette solution containing in 130 mM Cs^+, 9 mM EGTA, 4 mM MgATP, 0.3 mM GTP, and in the presence 5 mM Ba^{2+} and 160 mM TEA extracellularly. a, Current (I_{Ba}) in response to a depolarization from -80 mV to -10 mV, with capacity and leak correction. Traces are indicated for control, 2.5% isoflurane equilibrated solution, and after 10 minutes recovery from anesthetic. b, Current voltage relation for the neuron studied in A. c and d, Capacity and leak corrected I_{Ba} in response to a more sustained depolarization from -80 mV to 0 mV, for control and the presence of 2.5% isoflurane (c) or 1.5% halothane (d). Open circles indicate the control response scaled by 0.63 for isoflurane and 0.50 for halothane. Recovery currents are indicated by the dotted traces.

Since the increase of 50 mM K^+ results in a sustained depolarization during the $[Ca^{2+}]_i$ transient experiments, we examined anesthetic action on more prolonged depolarizations of 9.5 seconds. In this setting, 2.5% isoflurane clearly caused fractionally greater depression of later I_{Ba} components. As evident from the difference current in Figure 6a, the isoflurane-sensitive I_{Ba} showed a small initial inactivation, followed by a sustained component with minimum inactivation. When 1 μM nicardipine was applied for comparison, the nicardipine-sensitive L-type channel component of IBa was primarily a non-inactivating current of smaller magnitude than the isoflurane-sensitive current component (Figure 6b). The sustained block of I_{Ba} by nicardipine is clearly compatible with marked depression of the plateau of the $[Ca^{2+}]_i$ transient by this agent.

DISCUSSION

In granule neurons, clearly VAs inhibit the cur-

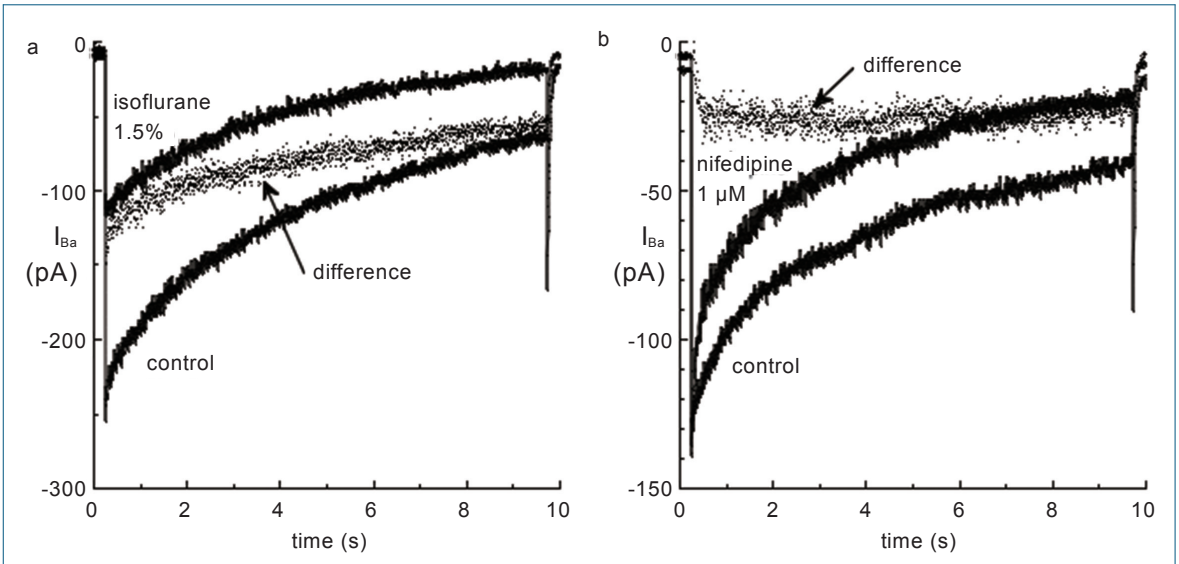

Figure 6. Capacity and Leak Corrected IBa in Response to a 9.5 s Depolarization from -80 mV to 0 mV. a, Control current and those in the presence of 2.5% isoflurane are shown. Dotted tracing indicates the difference current obtained by subtraction of the isoflurane from control current, and represents the current inhibited by isoflurane. Recovery to within 80% of control is omitted for clarity. b, Control current and that following addition of 1 μM nifedipine are shown. Dotted tracing indicates the difference current obtained by subtraction of nifedipine response from control current.

rents through VGCCs, the Ca^{2+} transient and the associated glutamate release in a concentration-dependent fashion. From the control studies it would appear that the K^+-induced depolarization mediates release of glutamate by the activation of VGCCs that mediate Ca^{2+} entry. When Ca^{2+} entry was altered by decreasing $[Ca^{2+}]_o$, glutamate release decreased in proportion to the decrease in the peak Ca^{2+} (25). While it could be argued that internal Ca^{2+} stores may be in rapid equilibrium with $[Ca^{2+}]_o$ and depleted when $[Ca^{2+}]_o$ is reduced, such an explanation seems less likely for the effects of altered $[Mg^{2+}]_o$. Since extracellular Mg^{2+} competes with Ca^{2+} in its electrostatic attraction to the negatively charged membrane surface or protein of the VGCC (32), altered $[Mg^{2+}]_o$ was used to change the available $[Ca^{2+}]_o$ for passage through the VGCCs (33). Increased $[Mg^{2+}]_o$ decreased the Ca^{2+} transient while halving of $[Mg^{2+}]_o$ increased it; changes that were mirrored by changes in glutamate release in a manner similar to that observed by directly altering $[Ca^{2+}]_o$ (25). For example, 10 mM $[Mg^{2+}]_o$ decreased peak $[Ca^{2+}]_i$ and glutamate release by a similar degree to that seen $[Ca^{2+}]_o$ to 400-600 μM. In contrast to the linear relation between of Ca^{2+} entry and glutamate release, more physiological studies suggest that transmitter release is a third or fourth order function of the presynaptic $[Ca^{2+}]_i$ elicited by an action potential (34, 35). The lower order dependence seen here probably reflects the fact that sustained depolarization prolongs Ca^{2+} entry, so that $[Ca^{2+}]_i$ equilibrates with the vesicular release machinery and glutamate release becomes a more linear function of Ca^{2+} entry.

The Ca^{2+} transient and the associated glutamate release in these neurons appear to be almost exclusively due to entry of Ca^{2+} via VGCC. Prior treatment with ryanodine or caffeine, in an attempt to deplete intracellular stores, did not alter the KCl-induced Ca^{2+} transient, or glutamate release (25). Specific VGCC blockade by drug and/or toxin is also consistent with virtually exclusive dependence on VGCC Ca^{2+} entry for the $[Ca^{2+}]_i$ increase that activates glutamate release. The use of Ctx-GVIA, nicardipine, and Aga-IVA was associated with similar decreases in the $[Ca^{2+}]_i$ transient peak. In other neurons, N-type current block was associated with only modest depression of glutamate release (22, 30) as observed in these experiments (Figure 2c); and P/Q-type channel blockade was associated with the greatest decrease in neurotransmitter release (36).

Electrophysiological studies (17, 37) have shown the presence of five distinct Ca channels with differing toxin sensitivity in CG neurons in culture. We used Ctx-GVIA, Aga-IVA, and nicardipine for the respective block of N-, P- and Q- and L-type VGCCs. Nicardipine block of the later plateau of $[Ca^{2+}]_i$ is consistent with the present and previous voltage clamp studies (38) showing that L-type current demonstrates less inactivation than the other VGCCs and would be expected to promote a more sustained Ca^{2+} transient. When combined nicardipine and Ctx-GVIA showed a slightly less than additive effect on $[Ca^{2+}]_i$ transients and plateau, probably indicating that some VGCCs in CG neurons have a mixed pharmacology, i.e. sensitivity to both Ctx-GVIA and DHP antagonists (39). When the three toxins were combined, the $[Ca^{2+}]_i$ transient was depressed to ~25% of control, which is similar to the remaining R-type current observed in CG neurons after similar blockade (17). Ongoing R-type VGCC activity permitted a small ongoing glutamate release suggesting it too supports neurotransmitter release (40).

It is important to note that the recorded glutamate release is occurring at the synaptic nerve endings or boutons, while the Ca^{2+} transient reflects Ca^{2+} increases in the cell body as well as the presynaptic endings; the recorded Ca^{2+} currents represents primarily Ca^{2+} entry in the cell body. There may be differences in the distribution of the different types of VGCCs between the cell body and the nerve endings and that difference may account for the minimal effect of Ctx-GVIA on glutamate (11% decrease), in spite of larger effect on Ca^{2+} transient and Ca^{2+} current (~45% decrease). In addition, while granule cell (CG) neurons are the most common neuron in the CNS, it is possible that GC neurons may be more or less susceptible to interventions or injury than cortical pyramidal cells, cerebellar Purkinje cells, other neurons, or glial cells.

These results demonstrate that therapeutic concentrations of VAs at physiological temperature markedly inhibit the elevation of $[Ca^{2+}]_i$ induced in cultured CG neurons by depolarization with 55 mM KCl. Figure 7 shows schematically the presynaptic ending and the Ca^{2+} induced release of glutamate. These effects are complemented by the depression of Ca^{2+} currents measured here by whole cell patch clamp experiments. The lower VA concentrations (75% halothane, 1.7% enflurane, 1.3% isoflurane, 2% sevoflurane) employed approximate human MAC (EC_{50}) values, at which movement in response to a surgical stimulus is inhibited in 50 percent of patients. For rats, the species from which the tissues were derived, these lower concentrations are approximately 0.8 MAC, and resulted in depression of the peak $[Ca^{2+}]_i$ transient to about 50% of the same day control level; 1.6 MAC caused even greater depression to about 20-30% of control. Both halothane and isoflurane caused substantial but incomplete inhibition of N-type and L-type Ca channel mediated entry into these neurons, since addition of the specific VGCC blocker for either channel still caused further blockade in the presence of VA. The effect of VAs on $[Ca^{2+}]_i$ peak and plateau in neurons pretreated with both nicardipine and Ctx-GVIA, or the I_{Ba} in similarly treated voltage clamped GCs, showed marked inhibition of the remaining response, suggesting that P/Q- and R-type VGCC are relatively sensitive to the VAs. After specifically blocking one or more of the various VGCC types, the VAs always had an effect on the remaining channels, consistent with our previous observation that the VA's inhibited the various types of VGCC to a similar extent when the specific associated α1 subunit was expressed in oocytes (α_{1A} =P/Q-type; α_{1B}=N-type; α_{1C}=L-type; α_{1E}=R-type)(19).

To delineate more clearly the relationship between VA actions on the $[Ca^{2+}]_i$ transient and glutamate release, the average fractional decrease in glutamate release was plotted versus the average decrease in the peak $[Ca^{2+}]_i$ transient for each anesthetic concentration studied (Figure 3d, e). Clearly, there is a parallel decrease, although for certain agents the relation between the peak of $[Ca^{2+}]_i$ and the glutamate release appears to be somewhat less than unity. Nevertheless, the effect of VAs is strikingly similar to the glutamate release/$[Ca^{2+}]_i$ transient relation observed other methods of blocking Ca^{2+} entry. Although some variation in the inactivation rate of I_{Ba} was observed in various neurons, the depression was similar for either isoflurane or halothane. The fractional inhibition of I_{Ba} at room temperature was somewhat less than the decrease in mea-

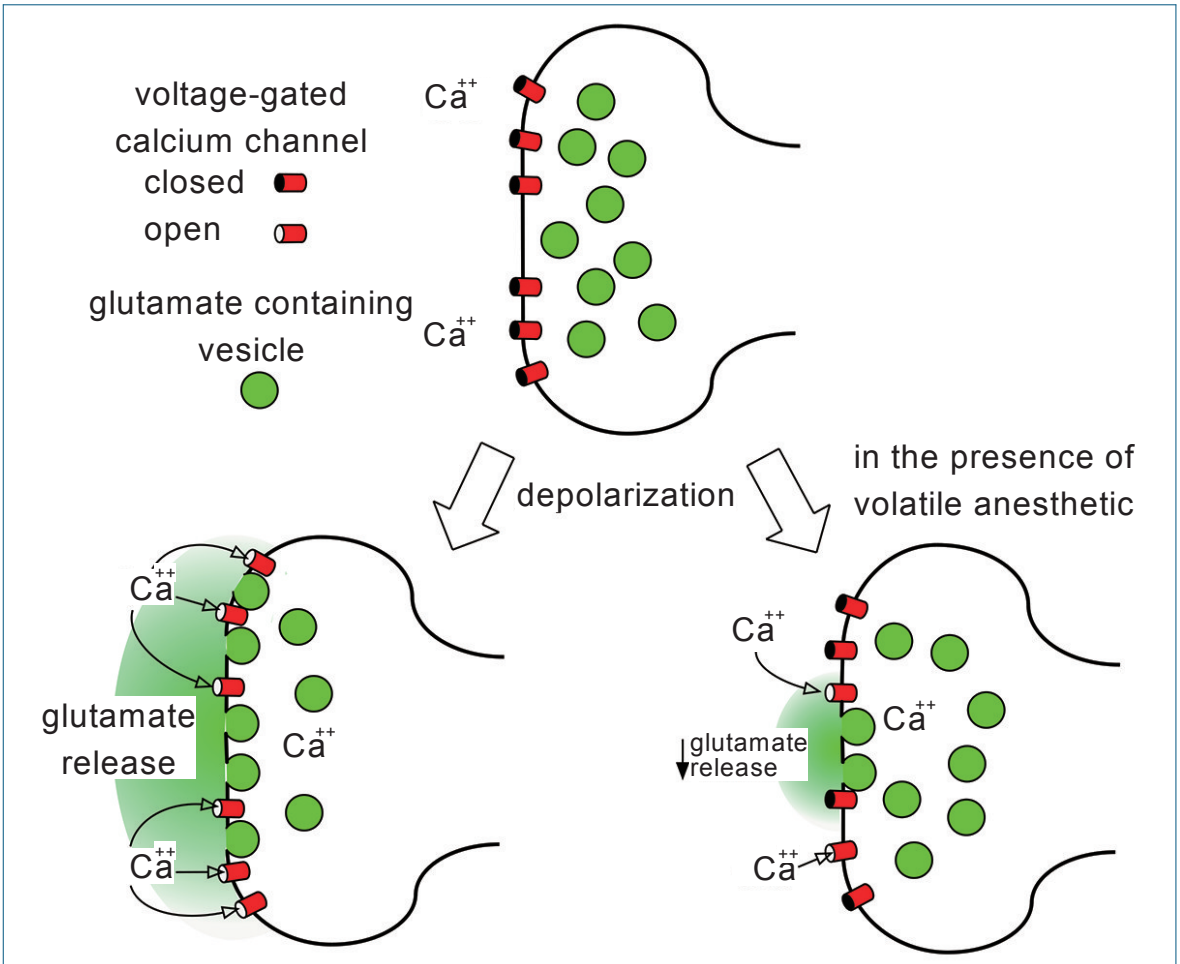

Figure 7. Schematic of Anesthetic Action on Ca^{2+} Entry and Glutamate Release.
Depolarization activates opening of presynaptic voltage-gated calcium channels (VGCC) permitting Ca^{2+} entry so that the vesicle release proteins (not shown) cause vesicle fusion and release of glutamate (lower left). By inhibiting VGCC opening, volatile anesthetics reduce Ca^{2+} entry and subsequent glutamate release (lower right).

sured Ca^{2+} peak transient, which may represent inherently different sensitivity at the two temperatures. The different charge-carrying ions could also contribute to variation, since with Ba^{2+} there will be no Ca^{2+}-dependent inactivation.

In vivo studies of experimental animals implicate inhibition of VGCC in certain behavioral components of the anesthetic state. Funnel-web spider toxin, which blocks P-type and other high-voltage gated Ca channels, can cause lethargy and stupor in mice (41); blockade of N- type channels by spinally administered - conotoxin MVIIA (SNX-111, ziconotide) has distinct antinociceptive actions (42). Non- specific VGCC blockade by Cd^{2+} as well as L-type channel block by verapamil have been found to enhance the anesthetic potencies of ethanol, pentobarbital, ketamine, and other anesthetics in mice (43, 44). While Mg^{2+} was employed as a tool to alter synaptic release, high serum [Mg^{2+}] reduce anesthetic requirement; four- fold increase of serum Mg^{2+} in rats reduces the anesthetic requirement for halothane by ~50% (45). The neuroactive agent riluzole (6- (trifluoromethoxy)benzothiazol- 2- amine) also decreases glutamate release and decreases anesthetic requirement (46), an effect that appears to be mediated in part by depression of Ca^{2+} entry by N- and P/Q- type VGCC (47).

VA-mediated decreases in Ca^{2+} entry and glutamate release may also have important implications beyond the analgesia and immobilization. Glutamate release is responsible in large part for the neuronal death that accompanies brain isch-

emia or anoxia, since blockade of glutamate release markedly reduces cell death (48). Glutamatergic neuronal activity is responsible for the bulk of brain energy utilization measures as glucose (13), primarily due to post-synaptic activation and its greater energy use (49). The glutamate activation of post- synaptic NMDA channels in particular may activate more sustained Ca^{2+} entry and intracellular accumulation. In so far as depression of glutamate release should decrease metabolic activity of the brain, presynaptic depressant effects could contribute to the VA-induced protection as it does for riluzole. The VA inhibition of VGCC- mediated Ca^{2+} entry could account in part for the decrease in glutamate release observed with ischemia (50), accounting in part for the apparent protective effect of VAs in some experimental models of brain ischemia (12, 51).

Depression of presynaptic Ca^{2+} entry itself may also contribute to neuronal protection within the central nervous system. For example, blockade of N-type VGCC in the CNS can reduce neuronal injury in models of both global (52) and focal cerebral ischemia (53). Such inhibition of CNS neuronal Ca channels may contribute to the potential neuroprotective effects of VAs, which have been described in a variety of previous studies and correlate in part to the decrease in cerebral metabolic activity (54- 57). Consistent with these observations are those demonstrating decreased glutamate release from anoxic cortical brain slices in the presence of halothane and enflurane (58) or from the ischemic hippocampal region of rats with isoflurane. It is evident from the tracings that after glutamate release was maximal, the $[Ca^{2+}]_i$ continued to increase due to the prolonged K^+-induced depolarization. Such ongoing increase in Ca^{2+} caused by the hyperkalemia that accompanies ischemia and hypoxia could contribute to neuronal cell death.

In summary, the commonly employed VAs at the equivalent concentrations used in this study had remarkably similar depressant actions on Ca^{2+} channel mediated entry into these CG neurons and the associated glutamate release. Such actions may contribute to certain protective actions of the VAs in the brain as well as behavioral and anesthetic effects of these drugs.

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