

General Anesthetics Do Not Affect Release of the Neuropeptide Cholecystokinin from Isolated Rat Cortical Nerve Terminals

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Background: **General anesthetics inhibit evoked release of classic neurotransmitters. However, their actions on neuropeptide release in the central nervous system have not been well characterized.**

Methods: **The effects of representative intravenous and volatile anesthetics were studied on the release of sulfated cholecystokinin 8 (CCK8s), a representative excitatory neuropeptide, from isolated rat cerebrocortical nerve terminals (synaptosomes). Basal, elevated KCl depolarization-evoked and veratridine-evoked release of CCK8s from synaptosomes purified from rat cerebral cortex was evaluated at 35°C in the absence or presence of extracellular Ca^{2+}. CCK8s released into the incubation medium was determined by enzyme-linked immunoassay after filtration.**

Results: **Elevation of extracellular KCl concentration (to 15–30 mM) or veratridine (10–20 μM) stimulated Ca^{2+}-dependent CCK8s release. Basal, elevated KCl- or veratridine-evoked CCK8s release was not affected significantly by propofol (12.5–50 μM), pentobarbital (50 and 100 μM), thiopental (20 μM), etomidate (20 μM), ketamine (20 μM), isoflurane (0.6–0.8 mM), or halothane (0.6–0.8 mM).**

Conclusions: **Clinically relevant concentrations of several classes of general anesthetics did not affect basal, KCl-evoked, or veratridine-evoked CCK8s release from isolated rat cortical nerve terminals. This is in contrast to the demonstrable effects of certain general anesthetics on the release of amino acid and catecholamine transmitters. These transmitter-specific presynaptic effects of general anesthetics suggest that anesthetic-sensitive presynaptic targets are not common to all transmitter classes.**

GENERAL anesthetics have both presynaptic and postsynaptic effects on synaptic transmission; they produce agent-specific effects on neurotransmitter release and the responses of neurons to neurotransmitters.[1] Most studies of the presynaptic actions of general anesthetics have focused on the classic transmitters: the amino acids (glutamate, γ-aminobutyric acid [GABA]) and acetylcholine. There is considerable evidence that the evoked release of these transmitters is depressed by both intravenous and volatile anesthetics,[1–4] which suggests that anesthetics target a process common to the release of all transmitters. However, few studies have analyzed the presynaptic actions of general anesthetics on peptidergic neurotransmission,[5,6] largely due to the technical difficulties involved in quantifying peptide release.[7] Given the conserved molecular mechanisms which are thought to control neurosecretion,[7–9] we hypothesized that general anesthetics should also inhibit neuropeptide release.

Cholecystokinin (CCK) was chosen as a representative neuropeptide for analysis since its release from central nervous system (CNS) nerve terminals has been studied in detail. Cholecystokinin is an 8-residue peptide (CCK8) found throughout the CNS. It is involved in many important functions, including memory, pain, appetite, and anxiety.[10–13] CCK8 is largely colocalized with classic transmitters in CNS terminals, where it is stored in morphologically distinct, large dense core vesicles. These vesicles are present throughout the presynaptic terminal and not at the active zones where classic transmitters contained in small synaptic vesicles are secreted. Sulfated CCK8 (CCK8s) is the predominant form in the CNS.[14] There are two CCK receptor subtypes in brain: CCK2 receptors mediate anxiety, panic attacks, satiety and pain, while the function of CCK1 receptors, which have limited distribution within the CNS, is poorly understood.[10,12,15]

The mechanism of CCK8s release has been studied extensively *in vivo*[11,16,17] and *in vitro*.[13,18–20] CCK8s release requires membrane depolarization and is dependent on extracellular Ca^{2+}. CCK8s coexists in brain areas and neurons that also contain other neurotransmitters, including catecholamines, acetylcholine, GABA, and glutamate.[13,21,22] These neurotransmitters can modulate CCK8s release from nerve endings, while CCK8s can modulate release of these neurotransmitters.[13,15] The coexistence of CCK8s with other neurotransmitters and its potential for modulation of transmission by other neurotransmitters implicated in anesthetic actions[1–3] suggest a potential role for CCK8s in general anesthesia. This study was designed to assess the direct presynaptic actions of representative general anesthetics on CCK8s release from nerve terminals isolated from rat cerebral cortex using a sensitive enzyme-linked immunoassay.

Materials and Methods

These studies were approved by Weill Medical College of Cornell University Institutional Animal Care and Use Committee (New York, New York).

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Materials

CCK8s was obtained from RBI (Natick, MA); Percoll was obtained from Pharmacia (Uppsala, Sweden); isoflurane was obtained from Abbott Laboratories (North Chicago, IL); and halothane (thymol-free) was obtained from Halocarbon Products (River Edge, NJ). Propofol was purchased from Aldrich Chemicals (Milwaukee, WI) or was a gift from AstraZeneca Pharmaceuticals (Wilmington, DE), and etomidate was a gift from Janssen Biotech n.v. (Olen, Belgium). Pentobarbital, thiopental, ketamine, polymeric L-glutamic acid (poly-Glu), 1-ethyl-3-dimethylaminopropylcarbodiimide hydrochloride, horseradish peroxidase (HRP)–labeled goat antirabbit IgG, rabbit anti-CCK8s IgG, bacitracin, Tween 20, veratridine, tetrodotoxin, ω-conotoxin MVIIC, dimethyl sulfoxide (DMSO), *o*-phenylenediamine, tablets for preparation of sodium carbonate-bicarbonate buffer pH 9.6, and HRP substrate solution (phosphate-citrate buffer with sodium perborate) were from Sigma Chemical Co. (St. Louis, MO). ω-Agatoxin IVA and ω-conotoxin GVIA were obtained from Alamone Labs (Jerusalem, Israel). All other chemicals were of analytical grade. Spectra/Por 12,000–14,000 dialysis membrane was obtained from Spectrum (Houston, TX); bovine serum albumin (fraction 5) was obtained from J.T. Baker (Phillipsburg, NJ); Nunc-immuno MaxiSorp flat-bottom modules (No. 469949), CoStar Spin-X centrifuge tube filters with 0.45-μm cellulose acetate membrane (Corning No. 8163), CoStar 96-well microtiter plates (No. 3797), and 0.4-ml polypropylene microcentrifuge tubes (No. 20170-326) were obtained from VWR Scientific Products (Bridgeport, NJ); and multiplates for filtration (No. MAFBNOB10) were obtained from Millipore (Bedford, MA).

Preparation of CCK8s-polyglutamic Acid

CCK8s was coupled to poly-Glu to form CCK8s-polyglutamic acid (CCK8s-poly-Glu).[23] A solution of poly-Glu (1.4 mg) in 1.3 ml water was added to a solution of 1-ethyl-3-dimethylaminopropylcarbodiimide hydrochloride (3.9 mg) in 0.39 ml water and stirred. A solution of CCK8s (0.2 mg) in 0.2 ml water was added over 45 s with mixing and left for 10 min at room temperature. The pH was adjusted to 6.0 with 0.27 ml sodium phosphate buffer, 1 M (pH 5.0), and the solution was stirred for 1 h at room temperature. The solution was diluted to 4.4 ml and dialyzed against 2 l water for 5 days at 4°C with a daily change of the dialysate. The dialyzed solution of CCK8s-poly-Glu conjugate was divided into aliquots, lyophilized, and stored at −70°C.

Preparation of Synaptosomes

Purified synaptosomes were prepared from rat cerebral cortex by the Percoll gradient method[24] as described.[25] The synaptosome fraction was washed from Percoll with HEPES-buffered medium (HBM) composed of the following: 130 mM NaCl, 3 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 10 mM D-glucose, and 20 mM HEPES, titrated to pH 7.4 with Tris base. Protein content was measured using the Bio-Rad Protein Assay Kit (Hercules, CA) based on the method of Bradford[26] using bovine serum albumin as a standard. Synaptosomes were stored as a pellet on ice and used within 5 h of preparation.

Filtration Release Assay

The effects of intravenous anesthetics on CCK8s release were assayed using a multifiltration system (Millipore Multiscreen Assay System; Millipore, Bedford, MA) by minor modifications of the procedure of Walaas.[27] All buffers contained 0.5 mg/ml bacitracin to minimize degradation of CCK8s.[28] Dilutions from stock solutions of propofol, thiopental, and etomidate in DMSO were made into HBM immediately prior to assays. DMSO as a vehicle at a final concentration of up to 0.5% (v/v) did not affect CCK8s release in control experiments (data not shown). Stock solutions of pentobarbital and ketamine were prepared in water and diluted into HBM.

Synaptosomes (120 μg protein in 60 μl HBM) plus any drugs for preincubation, and HBM (60 μl) containing drugs and/or the indicated secretogogues (15 or 30 mM final KCl; 10 or 20 μM veratridine) were preincubated in wells of separate plates (96-well microtiter plates, CoStar, No. 3797; VWR Scientific Products) with shaking for 5 min at 35°C. Reactions were initiated by transfer of synaptosomal aliquots to HBM aliquots (usually in triplicate or quadruplicate). After 5 min, reactions were stopped by transfer of synaptosomal suspensions to a third 96-well plate containing 15 μl EGTA, 100 mM, in HBM (final concentration approximately 10 mM). Synaptosomes were separated from incubation medium rapidly by vacuum filtration of samples through Millipore multiscreen filtration 96-well plates.

Centrifugation Release Assay

The effects of volatile anesthetics on CCK8s release were assayed using a centrifugation assay that allowed incubations in closed containers with minimal head space to reduce anesthetic losses. This assay was also used in control experiments to determine the effects of Ca^{2+} channel blockers on CCK8s release. Volatile anesthetics were prepared as saturated solutions in HBM (10–12 mM) at room temperature. Required volumes were diluted into 0.4-ml polypropylene microcentrifuge tubes containing synaptosomes. Synaptosomes (100–150μg in 330 μl HBM) were preincubated for 5 min at 35°C with or without volatile anesthetics in capped tubes. Ca^{2+} channel blockers prepared in HBM were added to synaptosomes in 1.5-ml polypropylene microcentrifuge tubes and preincubated at 35°C for 5 min. Concentrated KCl or veratridine (20 μl) was added through the cap with a Hamilton microsyringe, and tubes were sealed quickly with several layers of Parafilm (American National Can Co., Menasha, WI).

After incubation for 5 min at 35°C with mixing, reactions were stopped with EGTA (final concentration 10 mM). Synaptosomes separated from incubation solution rapidly from low-speed centrifugation for 30 s in CoStar Spin-X centrifuge tube filters (VWR Scientific Products) with an additional GF/B glass fiber filter layer.

CCK8s Quantification by Antigen Competition Enzyme-linked Immunoassay

CCK8s was quantified by enzyme-linked immunoassay[23,29] with minor modifications.

Solutions. The solutions used were as follows: (1) antigen-coating solution, 50 mM sodium carbonate–bicarbonate buffer, pH 9.6; (2) blocking solution, 20 mM Tris HCl, pH 7.4, 0.1% (w/v) BSA, 0.05% (v/v) Tween 20; (3) washing solution, blocking solution plus 0.15 mM NaCl; (4) substrate solution, 0.1 M phosphate–citrate buffer, pH 5.0, 0.03% (w/v) sodium perborate; and (5) stop solution, 4 N H_2SO_4.

Procedure. CCK8s-poly-Glu was dissolved in antigen-coating solution to 2 μg/100 ml (calculated for CCK8s content), and 200 μl of this solution was placed in each well of a Nunc-immuno MaxiSorb module (VWR Scientific Products) mounted in a frame. The plate with lid was incubated for 2 h at room temperature. The plate was rinsed twice with 200 μl blocking solution, filled again with 375 μl of this solution and left for at least 1 h at room temperature, and then rinsed thrice with 200 μl washing solution, which was completely decanted on a paper towel. Samples (60–120 μl synaptosome incubation medium) containing CCK8s were added to wells. Standard dilutions of pure CCK8s in duplicate in the same buffer were added to separate wells as standards. Concentrated washing solution was added to each well to achieve final concentrations of 0.15 M NaCl, 0.1% BSA, and 0.05% (v/v) Tween 20 as in the washing solution. An appropriate dilution of anti-CCK8s antibody in washing solution was added, and the covered plate was incubated overnight (12–16 h) at 4°C. Wells were washed five times over 30 min with washing solution, following which 200 μl HRP-conjugated goat antirabbit IgG (1:1000 dilution in washing solution) was added to each well. Covered plates were incubated for 2 h at 35°C. Wells were rinsed five times with washing solution, two times with 20 mM Tris HCl, pH 7.4, and two times with substrate solution. The HRP reaction was initiated by addition of 200 μl *o*-phenylenediamine (final concentration 0.4 mg/ml) in substrate solution to each well. Plates were incubated at room temperature for 30–45 min in the dark, and reactions were stopped by addition of 50 μl H_2SO_4, 4 N. Absorbance at 492 nm was measured using a Bio-Rad Microplate Reader (model 3550). Release of CCK8s is expressed as fmol CCKs/mg synaptosomal protein for 10 min, which includes 5 min of preincubation and 5 min of stimulation. In figures 1–3, CCK8s release was normalized to the mean basal release of CCK8s in HBM in the absence of added $CaCl_2$ (400–600 fmol/mg synaptosomal protein).

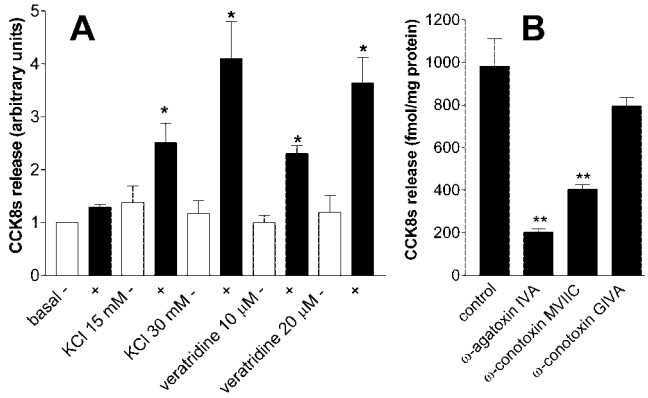

Fig. 1. Stimulus-evoked release of CCK8s from rat cortical synaptosomes. (*A*) Open bars: 0.1 mM EGTA with no added $CaCl_2$ (−); filled bars: 1.3 mM $CaCl_2$ (+). Results were normalized to control release in the absence of $CaCl_2$ (mean ± SD in arbitrary units). Data shown are from two to three experiments with each condition analyzed in quadruplicate. *$P < 0.05$ *versus* respective 0.1 mM EGTA with no added $CaCl_2$ control by Student *t* test. (*B*) Effects of Ca^{2+} channel antagonists on 30 mM KCl-evoked CCK8s release. Data (mean ± SD of one experiment analyzed in triplicate) are shown as Ca^{2+}-dependent release calculated by subtracting Ca^{2+}-independent release (0.1 mM EGTA with no added Ca^{2+}) from total release (plus 1.3 mM Ca^{2+}). Control: no toxins, 1 μM ω-agatoxin IVA, 5 μM ω-conotoxin MVIIC, or 5 μM ω-conotoxin GVIA. **$P < 0.001$ *versus* control by analysis of variance with Dunnett multiple comparison test.

Analysis of Anesthetic Concentrations

Free propofol concentrations in assay mixtures were determined by high-performance liquid chromatography[30]; the concentrations of propofol refer to the measured free concentrations. Initial and final volatile anesthetic concentrations in assay mixtures were determined by gas chromatography following extraction into n-heptane.[31] The extract (5 μl) was injected into a gas chromatograph (GC-8A; Shimadzu Corp., Kyoto, Japan) equipped with a thermal conductivity detector. Separation was achieved on a 1.8 m/6 mm ID glass column packed with Porapack Q (Supelco, Bellefonte, PA). The column temperature was 210°C, the injector temperature was 230°C, and carrier gas (He) flow was 40 ml/min. Volatile anesthetic concentrations refer to the average measured concentrations; there were no significant differences between the initial and final concentrations.

Statistical Analysis

Values are expressed as mean ± SD. Statistical significance was assessed by two-tailed Student *t* test or analysis of variance (ANOVA) with the Newman-Keuls multiple range test or Dunnett multiple comparison test using GraphPad Prism, version 2.01 (GraphPad Software, Inc., San Diego, CA); $P < 0.05$ was considered statistically significant.

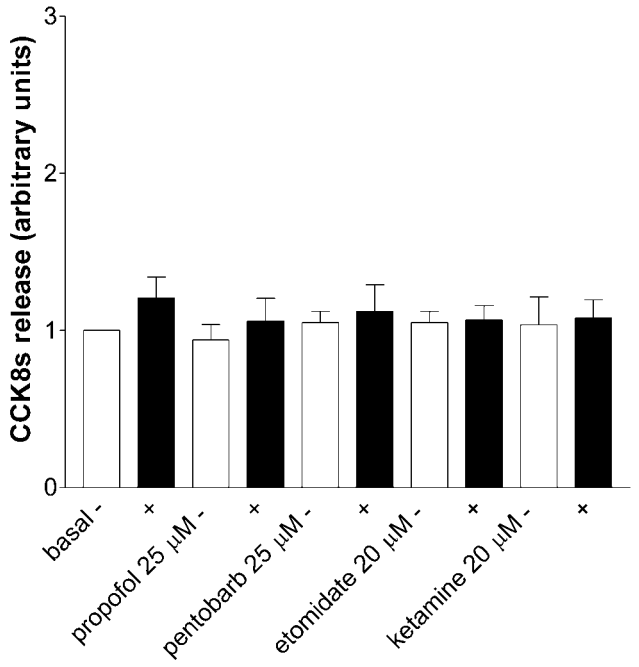

Fig. 2. Effects of intravenous general anesthetics on basal release of CCK8s. Open bars: 0.1 mM EGTA with no added $CaCl_2$ (−); filled bars: 1.3 mM $CaCl_2$ (+). Results were normalized to control release in the absence of $CaCl_2$ (mean ± SD in arbitrary units). Data shown are from two to three experiments with each condition analyzed in quadruplicate. There were no significant differences between anesthetic-treated and control synaptosomes by Student *t* test ($P > 0.05$).

Results

Characterization of CCK8s Release

Content of CCK8s in synaptosomes prepared from rat cerebral cortex was 6.7 ± 1.9 pmol/mg synaptosomal protein (n = 12). Basal (spontaneous) release in the absence of $CaCl_2$ was 400–600 fmol/mg synaptosomal protein over 10 min. Basal release was not increased significantly by exposure to 1.3 mM $CaCl_2$ (fig. 1A). The efflux of CCK8s evoked by elevated KCl or veratridine was concentration dependent and Ca^{2+} dependent. Release was increased threefold to fourfold by 30 mM KCl or by 20 μM veratridine. In control experiments, known P/Q-type Ca^{2+} channel antagonists inhibited KCl-evoked release, while an N-type Ca^{2+} channel antagonist was ineffective (fig. 1B). The specific Na^{+} channel antagonist tetrodotoxin completely inhibited veratridine-evoked release (see below).

Effects of Intravenous Anesthetics on CCK8s Release

Propofol (12.5–50 μM), pentobarbital (50–100 μM), thiopental (20 μM), etomidate (20 μM), or ketamine (20 μM) did not affect basal (fig. 2), 30 mM KCl-evoked (fig. 3), or 20 μM veratridine-evoked (fig. 4) release of CCK8s. Similar results were obtained whether synaptosomes were preincubated with the anesthetic for 5 min before addition of secretogogue, or anesthetic and secretogogue were added simultaneously (data not shown). Potentiation or inhibition of release was not observed when submaximal stimuli (15 mM KCl or 10 μM veratridine) were used (data not shown).

Effects of Volatile Anesthetics on CCK8s Release

Isoflurane or halothane at 0.6–0.8 mM (approximately 2 times minimum alveolar concentration [MAC]) did not affect basal or stimulus-evoked release of CCK-8s (figs. 5 and 6).

Discussion

Neither basal nor evoked release of the excitatory neuropeptide CCK was affected by clinically relevant concentrations of a number of intravenous and volatile general anesthetics. We employed a specific and sensitive enzyme-linked immunoassay to quantify CCK release from isolated rat cortical nerve terminals (synaptosomes). Synaptosomes are pinched-off nerve terminals prepared by gentle homogenization and subcellular fractionation of nervous tissue.[32] They represent the most accessible experimental system for the study of the biochemical and functional properties of presynaptic terminals. Our observations suggest that neither the ion chan-

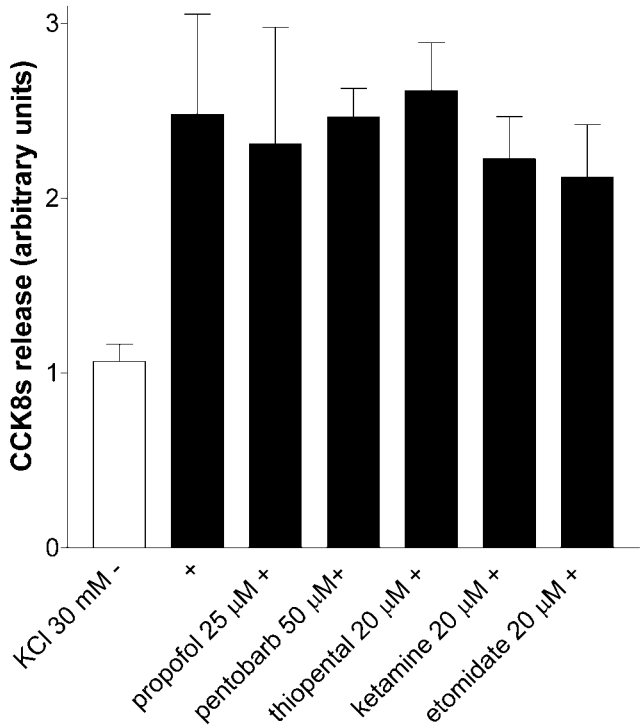

Fig. 3. Effects of intravenous anesthetics on KCl-evoked release of CCK8s. Open bar: KCl-evoked release with 0.1 mM EGTA and no added $CaCl_2$ (−); filled bars: KCl-evoked release with 1.3 mM $CaCl_2$ (+). Results were normalized to basal release in the absence of $CaCl_2$ (mean ± SD in arbitrary units). Data shown are from three to five experiments with each condition analyzed in quadruplicate. There were no significant differences in KCl-evoked release between anesthetic-treated and control synaptosomes by analysis of variance ($P > 0.05$).

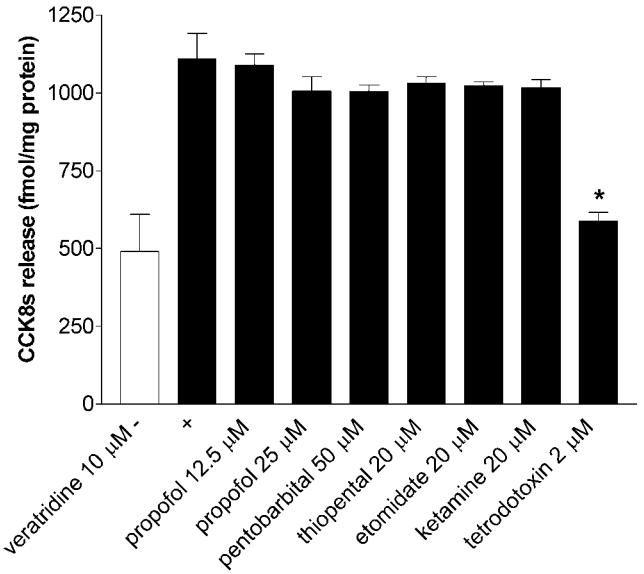

Fig. 4. Effects of intravenous anesthetics on veratridine-evoked release of CCK8s. Open bar: veratridine-evoked release with 0.1 mM EGTA and no added $CaCl_2$ (−); filled bars: veratridine-evoked release with 1.3 mM $CaCl_2$ (+). The data shown are from a single representative experiment (mean ± SD of triplicate assays). There were no significant differences in veratridine-evoked release between anesthetic-treated and control synaptosomes by analysis of variance ($P > 0.05$; n = 3). *$P < 0.05$ *versus* control veratridine-evoked release.

nels that couple depolarization of CCK-containing nerve terminals to Ca^{2+} influx nor the molecular machinery that couples this increase in Ca^{2+} to vesicle exocytosis is sensitive to concentrations of general anesthetics, which significantly affect the release of other transmitters under comparable conditions.

In agreement with previous studies, elevated KCl or veratridine evoked CCK release in a Ca^{2+}-dependent manner. Previous studies have analyzed CCK release from nerve terminals isolated from guinea pig[19] and rat hippocampus[20] and rat cerebral cortex.[33,34] Verhage *et al.*[20] reported basal release of approximately 150 fmol CCK/mg protein/3-min assay (50 fmol/mg protein/min), compared to our value of approximately 500 fmol CCK/mg protein/10-min assay (50 fmol/mg protein/min), which was increased approximately 2.3-fold by 30 mM KCl, compared to a stimulation of threefold to fourfold by 30 mM KCl in our experiments. Synaptosomes are too small for electrical field stimulation; transmitter release must be stimulated pharmacologically. We used two secretogogues that activate secretion by distinct mechanisms. Step elevations of extracellular KCl induce a "clamped" depolarization of the synaptosomal plasma membrane above the threshold for activation of voltage-gated Ca^{2+} channels present in the presynaptic terminal. KCl-evoked release is sensitive to P/Q-type Ca^{2+} channel blockade (Leenders *et al.*[34] and fig. 1A) but resistant to Na^+ channel blockade (*e.g.*, with tetrodotoxin). Na^+ channel activation by veratridine, a neurotoxin that inhibits channel inactivation, leads to prolonged synaptosome depolarization and influx of Ca^{2+}. Since Na^+ channel activation is required, release is tetrodotoxin-sensitive (figs. 4 and 6).[32] CCK release activated either by elevated KCl or by veratridine was not affected by general anesthetics, which suggests that the Na^+ and Ca^{2+} channel subtypes coupled to CCK release are insensitive. The interpretation of these studies must consider that artificial secretogogues are required to evoke transmitter release from isolated nerve terminals. Chemical stimulation may not accurately reproduce action potential-evoked release, which could result in underestimation of the sensitivity of neuropeptide release to anesthetics. Previous studies[34] and our control studies indicate that CCK release from rat cortical nerve terminals evoked with 30 mM KCl is inhibited by the P/Q-type Ca^{2+} channel antagonists ω-agatoxin IVA and ω-conotoxin MVIIC, and we showed that veratridine-evoked release is sensitive to the Na^+ channel antagonist tetrodotoxin. Together, these findings indicate that this assay is sensitive to blockade of presynaptic Ca^{2+} and/or Na^+ channels. Nevertheless, it is possible that the stimuli used to evoke CCK from isolated nerve terminals do not mimic action potential–evoked release and thus do not accurately reflect their sensitivity to anesthetics. Our findings do not rule out anesthetic actions on synaptic transmission by CCK mediated by mechanisms other than direct effects on release or on transmission by peptides other than CCK, such as those demonstrated in mollusks.[35]

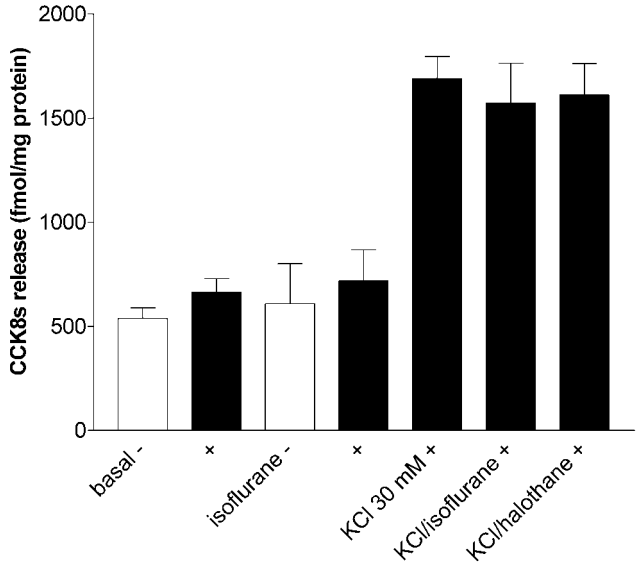

Fig. 5. Effects of volatile anesthetics on basal and KCl-evoked release of CCK8s. Open bars: 0.1 mM EGTA with no added $CaCl_2$ (−); filled bars: 1.3 mM $CaCl_2$ (+). The indicated assays contained 30 mM KCl, 0.60 mM isoflurane, and/or 0.65 mM halothane, respectively. The data shown are from a single representative experiment (mean ± SD of triplicate assays). There were no significant differences in KCl-evoked release between anesthetic-treated and control synaptosomes by analysis of variance ($P > 0.05$; n = 3).

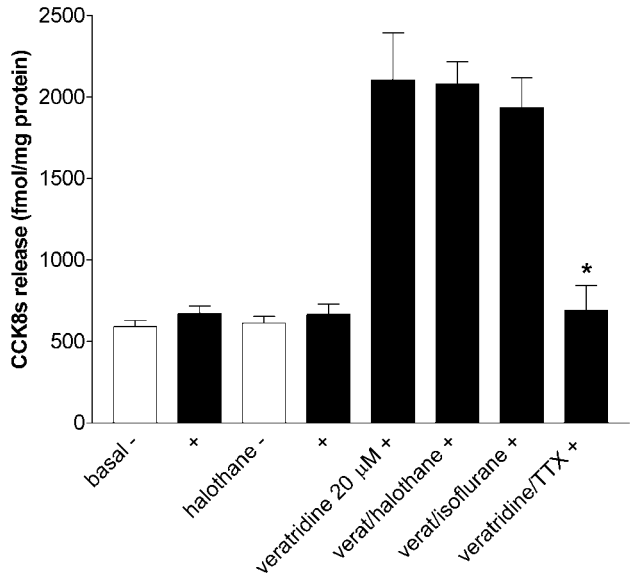

Fig. 6. Effects of volatile anesthetics on basal and veratridine-evoked release of CCK8s. Open bars: 0.1 mM EGTA with no added $CaCl_2$(−); filled bars: 1.3 mM $CaCl_2$ (+). The indicated assays contained 20 μM veratridine, 0.80 mM halothane, 0.70 mM isoflurane, and/or 2 μM tetrodotoxin. The data shown are from a single representative experiment (mean ± SD of triplicate assays). There were no significant differences in veratridine-evoked release between anesthetic-treated and control synaptosomes by analysis of variance ($P > 0.05$; n = 3). *$P < 0.05$ *versus* control veratridine-evoked release.

The fundamental mechanisms underlying transmitter release are largely conserved among the various classes of neurotransmitters.[7–9] Vesicles are released by Ca^{2+}-dependent fusion with the plasma membrane upon Ca^{2+} influx utilizing the highly conserved SNARE (soluble N-ethylmaleimide-sensitive fusion protein [NSF]-attachment protein [SNAP] receptor) core complex proteins syntaxin, synaptobrevin, and SNAP-25, as well as the Ca^{2+}-binding protein synaptotagmin. Genetic evidence for anesthetic effects on SNARE proteins was provided by a recent screening of existing mutants of the nematode *C. elegans* for alterations in sensitivity to volatile anesthetics, which identified mutations in all three components of the core fusion complex.[36] These findings imply that inhibition of transmitter release by general anesthetics, which is observed for many agents at clinical concentrations,[3] should be observed for all transmitter classes, if these mechanisms are shared by all transmitter classes. The results reported here indicate that this generalization does not hold for the excitatory neuropeptide cholecystokinin. Thus, concentrations of both volatile and intravenous anesthetics which inhibit depolarization-evoked release of glutamate (*e.g.*, isoflurane IC_{50} = 0.50 mM for inhibition of veratridine-evoked release in rat cortex),[25,37–39] GABA,[4] and acetylcholine[40] from small synaptic vesicles had no significant effect on CCK release from large dense core vesicles. This difference suggests that the mechanism involved in the presynaptic actions of general anesthetics is not a common property of all transmitter classes. Since classic transmitters are often colocalized with neuropeptides in the same nerve terminals (*e.g.*, GABA and CCK in cerebral cortex),[7] it is possible that general anesthetics selectively affect the release of the transmitters contained within small synaptic vesicles without affecting release of neuropeptides contained within dense core vesicles from the same terminals. Whether release of other neuropeptides is similarly resistant to general anesthetics will require further study.

Neurotransmitter release involves many steps, from action potential invasion of the presynaptic terminal; depolarization; ion channel gating; Ca^{2+} influx; vesicle translocation, docking and priming; Ca^{2+}-release coupling; vesicle fusion/exocytosis; to vesicle endocytosis. Each of these processes is modulated by repetitive activity, trophic factors, and presynaptic receptors. Certain differences in the molecular and cellular mechanisms that underlie the exocytotic release of fast transmitters (amino acids, monoamines) and slow transmitters (neuropeptides) have been described which could result in their differential sensitivities to general anesthetics. Neurons possess two types of secretory vesicles that undergo Ca^{2+}-dependent exocytosis with distinct kinetic differences despite their overall similar biochemical compositions.[7] Classic transmitters are released by fast exocytosis from small synaptic vesicles which cluster at active zones, whereas neuropeptides are released by fusion of large dense core vesicles with slower kinetics at sites away from the active zone.[9,41] Fast transmitters are released at active zones by relatively high local concentrations of Ca^{2+} (Ca^{2+} microdomains) produced by close physical coupling to specific Ca^{2+} channel subtypes, while neuropeptide release occurs by a more delocalized bulk phase increase in nerve terminal Ca^{2+} as a result of repetitive, high-frequency stimulation.[19,42] The apparent Ca^{2+} sensitivity of amino acid transmitter release from small synaptic vesicles is lower than that of neuropeptide release from large dense core vesicles[19] (but see Bollmann *et al.*[43]), which suggests the involvement of distinct Ca^{2+} sensors. Release of different classes of transmitters may also be coupled to distinct Ca^{2+} channel subtypes; release from small synaptic vesicles was coupled to N- and P-type Ca^{2+} channels, whereas CCK release from large dense core vesicles was coupled primarily to P/Q-type Ca^{2+} channels.[12,34] Neuropeptides are packaged into large dense core vesicles in the *trans*-Golgi network, whereas small synaptic vesicles are loaded by specific transporters in the terminal. Although exocytosis of both small and dense core vesicles involves the highly conserved SNARE protein machinery for fusion/exocytosis,[9,44,45] these different properties are consistent with subtle differences in their regulatory components, such as differential expression of synaptotagmin isoforms[46] and CAPS,[47] which is expressed on large dense core, but not small synaptic, vesicles. One or more of

these differences may be crucial in conferring sensitivity to general anesthetics of release of classic transmitters such as glutamate from small synaptic vesicles and insensitivity to anesthetics of the release of CCK, and possibly other transmitters, from large dense core vesicles.

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