Anesthesiology
2000; 92:529-41

Differential Effects of Anesthetic and Nonanesthetic Cyclobutanes on Neuronal Voltage-gated Sodium Channels

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Background: **Despite their key role in the generation and propagation of action potentials in excitable cells, voltage-gated sodium (Na^+) channels have been considered to be insensitive to general anesthetics. The authors tested the sensitivity of neuronal Na^+ channels to structurally similar anesthetic (1-chloro-1,2,2-trifluorocyclobutane; F3) and nonanesthetic (1,2-dichlorohexafluorocyclobutane; F6) polyhalogenated cyclobutanes by neurochemical and electrophysiologic methods.**

Methods: **Synaptosomes (pinched-off nerve terminals) from adult rat cerebral cortex were used to determine the effects of F3 and F6 on 4-aminopyridine– or veratridine-evoked (Na^+ channel–dependent) glutamate release (using an enzyme-coupled spectrofluorimetric assay) and increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) (using ion-specific spectrofluorimetry). Effects of F3 and F6 on Na^+ currents were evaluated directly in rat lumbar dorsal root ganglion neurons by whole-cell patch-clamp recording.**

Results: **F3 inhibited glutamate release evoked by 4-aminopyridine (inhibitory concentration of 50% [IC_{50}] = 0.77 mM [~ 0.8 minimum alveolar concentration (MAC)] or veratridine (IC_{50} = 0.42 mM [~ 0.4 MAC]), and veratridine-evoked increases in $[Ca^{2+}]_i$ (IC_{50}= 0.5 mM [~ 0.5 MAC]) in synaptosomes; F6 had no significant effects up to 0.05 mM (approximately twice the predicted MAC). F3 caused reversible membrane potential–independent inhibition of peak Na^+ currents (70 ± 9% block at 0.6 mM [~ 0.6 MAC]), and a hyperpolarizing shift in the voltage-dependence of steady state inactivation in dorsal root ganglion neurons (−21 ± 9.3 mV at 0.6 mM). F6 inhibited peak Na^+ currents to a lesser extent (16 ± 2% block at 0.018 mM [predicted MAC]) and had minimal effects on steady state inactivation.**

Conclusions: **The anesthetic cyclobutane F3 significantly inhibited Na^+ channel–mediated glutamate release and increases in $[Ca^{2+}]_i$. In contrast, the nonanesthetic cyclobutane F6 had no significant effects at predicted anesthetic concentrations. F3 inhibited dorsal root ganglion neuron Na^+ channels with a potency and by mechanisms similar to those of conventional volatile anesthetics; F6 was less effective and did not produce voltage-dependent block. This concordance between anesthetic activity and Na^+ channel inhibition supports a role for presynaptic Na^+ channels as targets for general anesthetic effects and suggests that shifting the voltage-dependence of Na^+ channel inactivation is an important property of volatile anesthetic compounds. (Key words: DRG neurons; glutamate release; intracellular calcium; Na^+ currents; synaptosomes; volatile anesthetics.)**

VOLTAGE-GATED ion channels have been considered to be insensitive to general anesthetics.[1] However, volatile anesthetics have substantial electrophysiologic effects on voltage-gated sodium (Na^+) channels in intact tissues,[2-5] and electrophysiologic analysis of cloned rat CNaIIa Na^+ channels (in cells transfected with a cDNA encoding the α subunit of the rat brain type IIa Na^+ channel) indicates significant inhibition of Na^+ currents[6] by clinical concentrations of volatile anesthetics. Actions of volatile anesthetics on presynaptic Na^+ channels coupled to glutamate release have also been shown in rat cortical synaptosomes.[7] Previous studies suggesting that Na^+ channels were relatively insensitive to general anesthetics used hyperpolarized holding potentials or peripheral invertebrate tissues,[8-10] which may have resulted in reduced apparent anesthetic sensitivity.

Structure-activity analysis of a series of polyhalogenated compounds identified agents that deviate from the Meyer-Overton correlation between anesthetic potency

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Received from the Department of Anesthesiology, Weill Medical College of Cornell University, New York, New York. Submitted for publication April 22, 1999. Accepted for publication September 29, 1999. Supported by National Institutes of Health grant GM 58055, Bethesda, Maryland (Dr. Hemmings) and a fellowship from the Norman and Rosita Winston Foundation in Biomedical Sciences, New York, New York (Dr. Ratnakumari). Presented in part at the annual meeting of the American Society of Anesthesiologists, Orlando, Florida, October 17-21, 1998, and the 28th annual meeting of the Society for Neuroscience, Los Angeles, California, November 7-12, 1998.

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and lipid solubility. Among compounds predicted to be anesthetics by the Meyer-Overton correlation were agents that produce general anesthesia (defined by minimum alveolar concentration [MAC] determinations); compounds that produce anesthesia at higher than predicted concentrations and convulsions (transitional compounds); and compounds that do not produce anesthesia ("nonimmobilizers") but produce amnesia, analgesia, and convulsions.[11,12] These structurally similar compounds with distinct anesthetic actions may be valuable in identifying molecular sites of anesthetic action. Sensitivity to anesthetic, but not to nonimmobilizer, compounds supports the relevance of a putative site to the production of the immobilizing aspect of anesthesia and *vice versa*.[11] Although they both partition into membranes, 1-chloro-1,2,2-trifluorocyclobutane (F3) is an effective anesthetic, but 1,2-dichlorohexafluorocyclobutane (F6) does not produce anesthesia or potentiate the anesthetic potency of desflurane[11,13] and induces convulsions in rats.[13,14] The ability of F6 to cause amnesia and convulsions suggests that it crosses the blood–brain barrier and reaches sufficient concentrations to affect neuronal targets, which appear to differ from targets relevant to anesthesia.

We analyzed the effects of these anesthetic (F3) and nonanesthetic (F6) polyhalogenated cyclobutanes on Na^+ channel function using neurochemical and electrophysiologic approaches. Presynaptic Na^+ channel function was assessed by measuring veratridine- and elevated KCl–evoked glutamate release and increases in intrasynaptosomal Ca^{2+} concentration ($[Ca^{2+}]_i$) in rat cerebrocortical synaptosomes (pinched-off nerve terminals). Na^+ currents were also assessed directly by whole-cell patch-clamp recording in isolated rat lumbar dorsal root ganglion (DRG) neurons. Anesthetic concentrations of F3 significantly inhibited Na^+ channel–dependent glutamate release and increases in $[Ca^{2+}]_i$ and blocked DRG Na^+ currents; F6 had no significant effects on Na^+ channel-dependent synaptosome functions and reduced effects on DRG Na^+ currents. These findings support the hypothesis that inhibition of neuronal Na^+ channels is involved in the immobilizing actions of volatile anesthetics.

Materials and Methods

Experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals, as approved by the Weill Medical College of Cornell University Institutional Animal Care and Use Committee.

Materials

Nicotinamide adenine dinucleotide phosphate ($NADP^+$), L-glutamate dehydrogenase (*Proteus* species), bovine serum albumin (BSA; essentially fatty acid free), 4-aminopyridine (4AP), veratridine, fura-2 acetoxymethyl ester, nifedipine, tetrodotoxin (TTX), collagenase (type IV), trypsin (type XII), and pronase E (type XIV) were obtained from Sigma Chemical Co. (St. Louis, MO); Percoll from Pharmacia (Uppsala, Sweden); β-phorbol 12,13-dibutyrate from LC Laboratories (Woburn, MA); F3 (1-chloro-1,2,2-trifluorocyclobutane) and F6 (1,2-dichlorohexafluorocyclobutane) from PCR (Gainesville, FL); Hank's balanced salt solution, fetal bovine serum, Dulbecco's modified eagle medium/F-12, penicillin, and streptomycin from GibcoBRL (Gaithersburg, MD); and ω-agatoxin IVA and ω-conotoxin GVIA from Alomone Labs (Jerusalem, Israel). All other chemicals were of analytic grade.

Isolation of Synaptosomes

Synaptosomes were prepared from cerebral cortices of adult Sprague-Dawley rats by the method of Dunkley *et al.*,[15] with minor modifications as described.[16] Purified synaptosomes were suspended in HEPES buffered medium (HBM; 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 5 mM $NaHCO_3$, 10 mM D-glucose, and 20 mM HEPES, *p*H 7.4 with NaOH), pelleted by centrifugation at 8,000*g* for 10 min, and stored on ice for up to 5 h until use.

Measurement of Glutamate Release

Release of endogenous glutamate was measured by an enzyme-linked fluorescence method.[17] Synaptosomal pellets (0.5 mg protein) were resuspended in 1.5 ml HBM plus 16 μM bovine serum albumin, 1 mM $NADP^+$, 100 U L-glutamate dehydrogenase, and 1.3 mM $CaCl_2$. Stirred samples were equilibrated at 37°C for 4 min in a spectrofluorometer cuvette, and data acquisition was started (excitation wavelength = 340 nm; emission wavelength = 460 nm). After recording basal fluorescence change (ΔF) for 60 s, F3 or F6 was added as saturated HBM, and ΔF was measured from 0 s to 60 s. Two hundred seconds after the addition of F3 or F6 (25–250 μl), secretogogue (6 μl of 0.25 M 4AP + 1.5 μl of 1 mM β-phorbol 12,13-dibutyrate, 3 μl of 10 mM veratridine, or 15 μl of 3 M KCl) was added, and the initial rate (0–60 s) of ΔF was measured. Longer prein-

cubation with cyclobutanes did not produce greater effects (data not shown). The fluorescence signal was calibrated by adding 5 nmol L-glutamate to the cuvette at the end of each experiment.

Glutamate release evoked by 4AP, veratridine, or KCl was measured to assess the involvement of presynaptic Na^+ channels as targets for drug actions. 4AP, a potassium (K^+) channel blocker, destabilizes membrane potential and causes repetitive spontaneous Na^+ channel-dependent (tetrodotoxin-sensitive) depolarizations, which mimic depolarization of nerve terminals by action potentials and lead to activation of Ca^{2+} channels and neurotransmitter release. Veratridine activates Na^+ channels directly and thereby depolarizes the plasma membrane, leading to Ca^{2+} channel activation and Na^+ channel-dependent (tetrodotoxin-sensitive) neurotransmitter release. Elevated extracellular KCl concentrations depolarize the plasma membrane by shifting the K^+ equilibrium potential above the threshold potential for activation of Ca^{2+} channels, leading to Ca^{2+} entry and Na^+ channel-independent (tetrodotoxin-insensitive) neurotransmitter release; Na^+ channels are inactivated.[18]

Measurement of Intrasynaptosomal $[Ca^{2+}]$

Synaptosome pellets were resuspended to 3 mg protein/ml in HBM containing 5 μM fura-2 acetoxymethyl ester (dissolved in dimethyl sulfoxide [DMSO]) and 40 μM bovine serum albumin and incubated for 30 min at 37°C. Loading was discontinued by the addition of 20 vol dye-free HBM. Synaptosomes were incubated at room temperature for an additional 30 min to complete dye hydrolysis, and the suspension was centrifuged at 10,000*g* for 10 min. The pellet was resuspended in HBM to 1 mg protein/ml, divided into 0.5-ml aliquots, centrifuged at 10,000*g* for 5 min, and stored on ice until use. For measurement of free $[Ca^{2+}]_i$, pellets were resuspended in 1.5 ml HBM and transferred to a stirred quartz cuvette at 37°C. After recording basal fluorescence for 100 s, 1.3 mM $CaCl_2$ was added, followed 200 s later by secretogogue (3 μl of 10 mM veratridine or 15 μl of 3 M KCl). Fluorescence was measured for another 200 s, and $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence intensity of fura-2 at an emission wavelength of 510 nm, with excitation wavelengths of 340 and 380 nm (switched every 2 s). In experiments with cyclobutanes, the assay was initiated directly by the addition of different volumes of saturated solutions of F3 or F6, followed by recording of their effects on basal fluorescence. Calibration was performed by determining the maximal (saturating $[Ca^{2+}]$ was obtained by lysis of the synaptosomes in 6.2 mM Triton X-100 [octylphenolpoly(ethyleneglycolether)$_n$]) and minimal fluorescence ratios (0 $[Ca^{2+}]$ was obtained by chelating free Ca^{2+} with 7.7 mM ethyleneglycol *bis*-[β-aminoethyl ether] N,N,N′,N′-tetraacetic acid plus 40 mM TRIS base). Fluorescence ratio was converted to $[Ca^{2+}]_i$ using software based on the method of Grynkiewicz *et al.*[19]

Synaptosomal $[Ca^{2+}]_i$ was determined at the same times before and after the addition of secretogogues as in glutamate-release experiments. Basal $[Ca^{2+}]_i$ was averaged over a 60-s period (30–90 s); resting $[Ca^{2+}]_i$ was averaged from 0–60 s after the addition of $CaCl_2$; and veratridine-stimulated increases in $[Ca^{2+}]_i$ were averaged for 60 s after the addition of secretogogue. KCl-evoked increases in peak $[Ca^{2+}]_i$ were measured between 0 and 30 s, and plateau $[Ca^{2+}]_i$ was measured from 30 to 60 s after KCl addition.

Isolation of Dorsal Root Ganglion Neurons

Intermediate-sized DRG neurons (15–40 μm diameter) were isolated from Sprague-Dawley rats aged 5–30 days by a modification of described methods.[20,21] Briefly, animals were anesthetized with 80% CO_2–20% O_2 and decapitated, and the DRGs were quickly removed and placed in oxygenated, ice-cold normal or Ca^{2+}-free Hank's balanced salt solution (1.3 mM $CaCl_2$, 5 mM KCl, 0.3 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 137.9 mM NaCl, 0.3 mM Na_2HPO_4, and 5.6 mM glucose) containing one or a combination of the following proteases (in wt/vol): trypsin (0.1–0.2%), collagenase (0.1%) or pronase E (0.05–1%), depending on age of animal (from 5–25 days) and also for control purposes. The different combinations of proteases or the age of animals did not affect the maximum Na^+ current or the effects of drugs. After enzyme incubation, ganglia were placed in D-MEM/F-12 containing 20% (vol/vol) fetal bovine serum and agitated using a Pasteur pipette. Dissociated cells were plated on polylysine-covered Petri dishes. Cells were used from a few hours after dissociation up to three days of primary culture in a 95% O_2–5% CO_2 incubator at 37°C. Culture medium consisted of D-MEM/F-12 with L-glutamine, 10% (vol/vol) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Patch-clamp Recording

Na^+ currents were studied using the whole-cell configuration of the patch-clamp recording technique, using a standard patch-clamp amplifier (Axopatch 200; Axon Instruments, Foster City, CA) controlled by commercially available software (pCLAMP; Axon Instruments)

on a standard personal computer. Patch-clamp pipettes were pulled from micropipette glass (Drummond, Broomall, PA) and filled with intracellular solution containing 10 mM NaCl, 140 mM cesium fluoride (CsF), and 5 mM HEPES, pH 7.2. The external solution was Hank's balanced salt solution plus 20 mM tetraethylammonium chloride, 5 mM HEPES, and 0.005 mM $LaCl_3$, pH 7.2. Under these conditions, tetrodotoxin-resistant channels, which are present in some DRG neurons, were blocked,[22] and only currents through tetrodotoxin-sensitive channels were recorded. This was verified by recordings in the presence of tetrodotoxin (1 μM), which blocked all Na^+ currents (data not shown). The cyclobutanes were applied *via* a glass-polytetrafluoroethylene perfusion system, with a superfusion pipette (flow rate 0.5–0.8 ml/min) close to the cell. Recordings were made at room temperature (22 ± 1°C). Cyclobutane concentrations reported are those in the recording chamber, which include a loss of 14% F3 and 28% F6 from the stock solutions. Currents were filtered at 5 kHz, digitized, and recorded to hard disk. Capacitive transients and series resistance were measured and compensated using the amplifier's internal compensation circuitry; active series-resistance compensation was used to compensate for 60–85% of the series resistance. Cells with currents greater than 6 nA or smaller than 1 nA or with leakage greater than 5% of maximal Na^+ conductance were rejected.

To estimate the application kinetics of F3 and F6, we routinely evaluated currents with a test pulse to +10 mV from a holding potential of −100 mV. With F3, significant effects were observed within 20 s of solution exchange; effects of F6 occurred more slowly. With both compounds, maximal effects were observed within 2 min of application, and no further changes in current were observed for up to 10 min. Therefore, the effects of F3 and F6 were measured after 2 min of application, when both compounds had apparently reached a steady state in the experimental system.

Cyclobutane Preparation and Quantification

Saturated cyclobutane solutions were freshly prepared by adding 90 μl cyclobutane to 30 ml buffer in an airtight container. Solutions were incubated overnight at room temperature with mixing. The concentrations of saturated solutions obtained at 22°C were ~9 mM F3 and 0.25 mM F6. Glutamate-release assays at high cyclobutane concentrations were initiated by addition of large volumes of cyclobutane solutions to the cuvette without recording basal ΔF. Cyclobutane concentrations in stock solutions and in each assay mixture for glutamate release and $[Ca^{2+}]_i$ experiments were determined by gas chromatography. Aliquots were withdrawn with a gas-tight microsyringe and extracted into 100 μl *n*-heptane, of which 5 μl was injected onto a gas chromatograph (GC-8A; Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector. Separation was achieved on a 1.8 m × 6 mm glass column packed with Porapak Q (Supelco, Bellefonte, PA). The column temperature was 210°C, the injector temperature was 230°C, and the carrier helium flow was 40 ml/min.

Statistical Analysis

Statistical differences between control and experimental values were determined by analysis of variance using the Fisher *post hoc* test. Concentration-effect data were analyzed for IC_{50} values by linear regression using data between 20 and 80% of the maximal response (Pharm/PCS Pharmacologic Calculation System, version 4.2; Springer Verlag, New York, NY). Values are expressed as the mean ± SD.

Electrophysiologic analysis was performed on-line with a second acquisition computer using pCLAMP6 software. Curve fits and statistical analysis were performed using commercially available software: Origin, pCLAMP Module for Origin (Microcal Software, Northampton, MA) and GraphPad InPlot (GraphPad Software, San Diego, CA).

Miscellaneous

Protein concentrations were determined by the Coomassie blue method[23] using bovine serum albumin as a standard.

Results

Glutamate Release

F3 (MAC, 0.99 mM)[11] inhibited 4AP-evoked glutamate release in a concentration-dependent manner (fig. 1), with an IC_{50} of 0.77 mM. F3 also inhibited veratridine-evoked glutamate release in a concentration-dependent manner (fig. 2), with an IC_{50} of 0.42 mM. F3 did not significantly affect elevated KCl-evoked (35 mM final) release up to 1.2 mM but was effective at a high concentration (69% inhibition at 2.4 mM; fig. 3).

F6 (predicted MAC = 0.022 mM)[11] up to 0.05 mM (~two times predicted MAC) did not significantly affect 4AP- or veratridine-evoked glutamate release (figs. 1 and 2). F6 potentiated KCl-evoked glutamate release by 58%

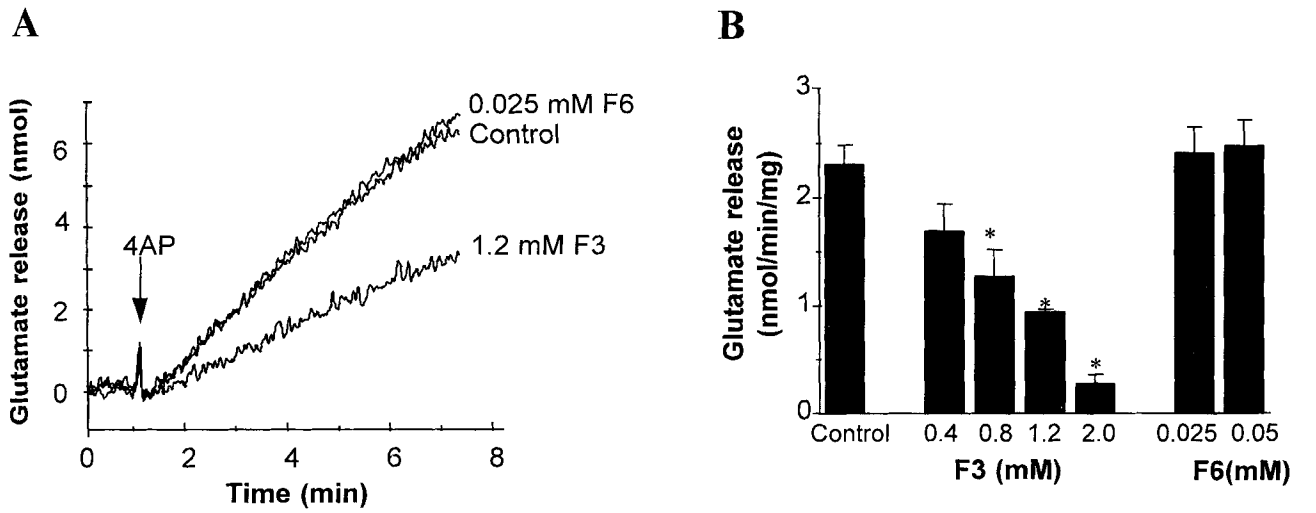

Fig. 1. Effects of F3 and F6 on 4-aminopyridine-evoked glutamate release from cortical synaptosomes. (*A*) Representative experiments. (*B*) Summary data expressed as mean ± SD (n = 3). **P* < 0.05 *versus* control (no cyclobutane) by analysis of variance with the Fisher *post hoc* test.

at 0.025 mM but had no significant effect at 0.05 mM (fig. 3), the highest concentration achievable in this assay.

Intrasynaptosomal [Ca^{2+}]

Values for $[Ca^{2+}]_i$ in the absence (154 ± 24 nM; n = 14) or the presence (290 ± 40 nM; n = 14) of 1.3 mM extracellular $CaCl_2$ agreed with previously reported values of 164 ± 17 nM and 369 ± 66 nM[24] and 170 nM and 354 nM.[25] Lower concentrations of F3 or F6 did not affect resting $[Ca^{2+}]_i$ before or after addition of extracellular Ca^{2+} (data not shown), although 2.4 mM F3 significantly enhanced resting $[Ca^{2+}]_i$ before (to 178 ± 25; $P < 0.05$; n = 4) and after (to 387 ± 28; $P < 0.05$; n = 4) addition of $CaCl_2$.

Addition of $CaCl_2$ and veratridine each resulted in a rapid increase in $[Ca^{2+}]_i$, which reached a plateau after 30–40 s (fig. 4). After the addition of 30 mM KCl (35 mM final), $[Ca^{2+}]_i$ rapidly increased to peak levels, which decreased to plateau levels in 30–60 s (fig. 5A). KCl-evoked increases in peak and plateau $[Ca^{2+}]_i$ were partially blocked by 1 μM nifedipine (L-type Ca^{2+} channel blocker; −27 and −23%, respectively) or by 0.2 μM ω-agatoxin IVA plus 0.1 μM

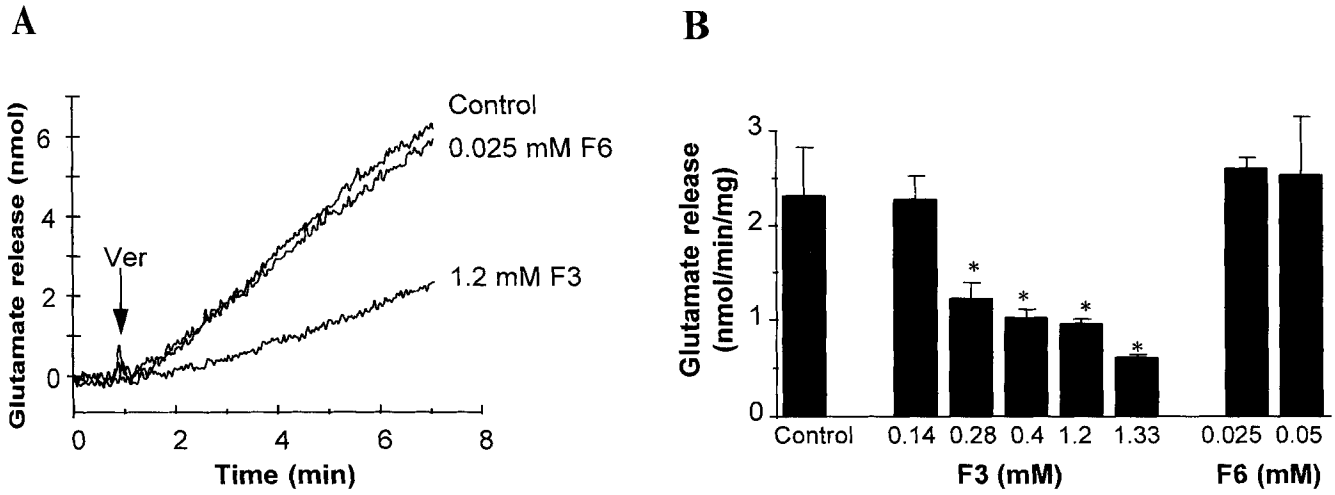

Fig. 2. Effects of F3 and F6 on veratridine-evoked glutamate release from cortical synaptosomes. (*A*) Representative experiments. (*B*) Summary data expressed as mean ± SD (n = 3). **P* < 0.05 *versus* control (no cyclobutane) by analysis of variance with the Fisher *post hoc* test. Ver = veratridine.

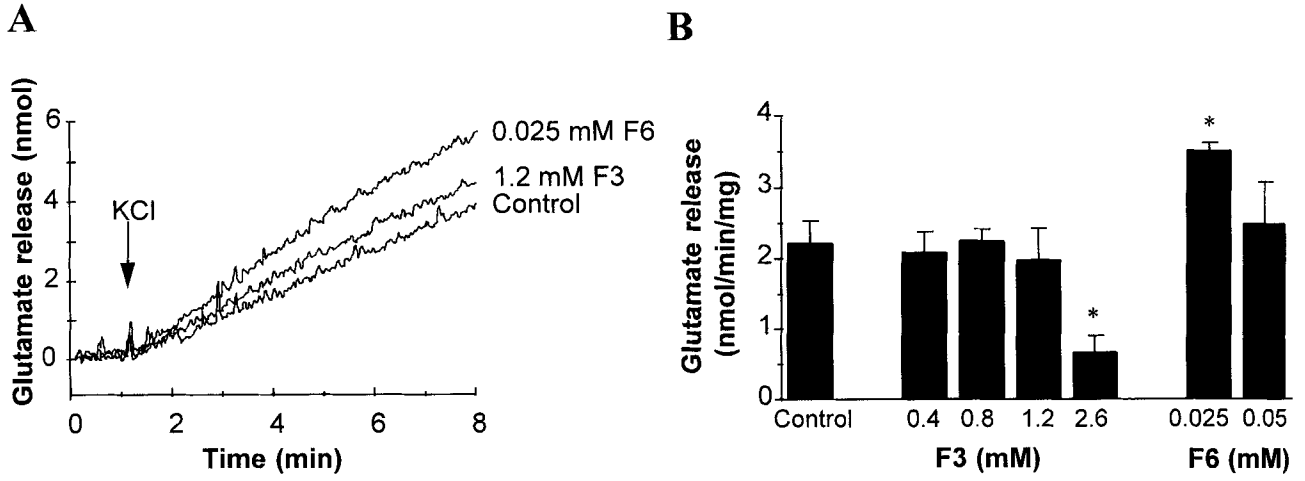

Fig. 3. Effects of F3 and F6 on KCl-evoked glutamate release from cortical synaptosomes. (*A*) Representative experiments. (*B*) Summary data expressed as mean ± SD (n = 3). *$P < 0.05$ *versus* control (no cyclobutane) by analysis of variance with the Fisher *post hoc* test.

ω-conotoxin GVIA (P/Q and N-type Ca^{2+} channel blockers;[26] −57 and −61%, respectively). Veratridine-evoked increases in $[Ca^{2+}]_i$ were completely blocked by 1 μM tetrodotoxin (data not shown).

F3 inhibited veratridine-induced increases in $[Ca^{2+}]_i$ with an IC_{50} of 0.5 mM (fig. 4). No significant effects of F3 up to 1.2 mM were observed on KCl-induced increases in either the peak or the plateau phases of $[Ca^{2+}]_i$ (figs. 5B and C); F3 at 2.4 mM significantly inhibited the peak increase of $[Ca^{2+}]_i$ (−28%; fig. 5B). F6 (up to 0.05 mM) did not significantly affect veratridine- or KCl-induced increases in $[Ca^{2+}]_i$ (figs. 4 and 5).

Dorsal Root Ganglion Na^+ Currents

Na^+ currents were elicited by stepping the resting membrane potential from −100 mV, at which channel inactivation is removed, to test potentials from −60 mV to +100 mV. This protocol evaluates the interactions of F3 or F6 with the resting (closed) and open states of the channel. F3 significantly inhibited peak Na^+ currents in a concentration-dependent manner (fig. 6A). Inhibition occurred within seconds of drug application and could be reversed by perfusion with anesthetic-free medium. In contrast, although F6 also inhibited peak current (fig. 6B), the effect was much less than that observed with

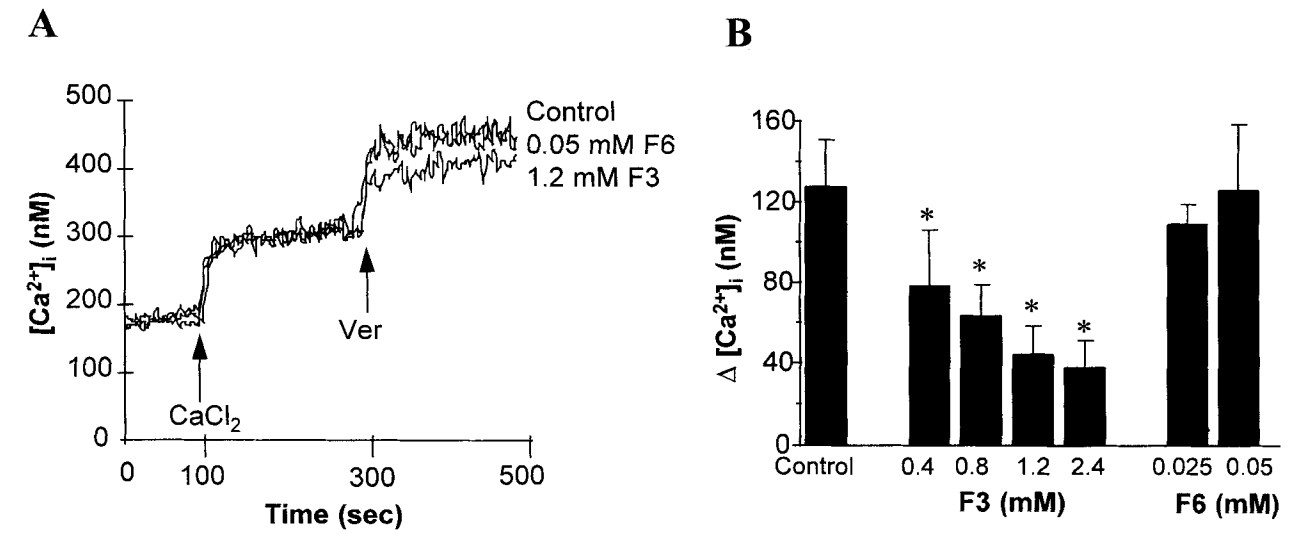

Fig. 4. Effects of F3 and F6 on veratridine-evoked increases in $[Ca^{2+}]_i$ in cortical synaptosomes. (*A*) Representative experiments. (*B*) Summary data expressed as mean ± SD (n = 4). *$P < 0.05$ *versus* control (no cyclobutane) by analysis of variance with the Fisher *post hoc* test.

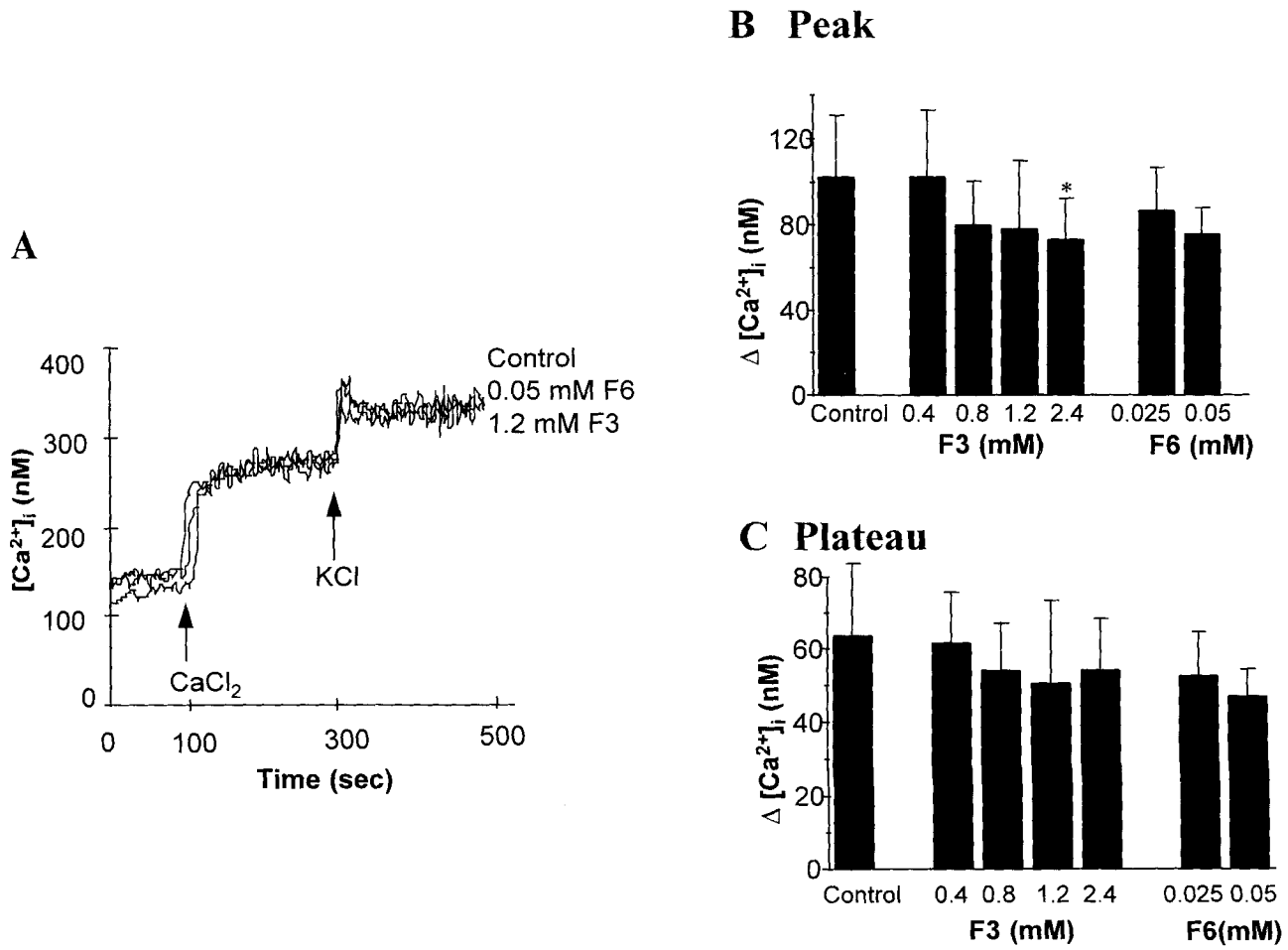

Fig. 5. Effects of F3 and F6 on KCl-evoked increases in peak and plateau phase $[Ca^{2+}]_i$ in cortical synaptosomes. (*A*) Representative experiments. (*B, C*) Summary data expressed as mean ± SD (n = 4). *$P < 0.05$ *versus* control (no cyclobutane) by analysis of variance with the Fisher *post hoc* test.

the equivalent MAC concentrations of F3 and was not reversible, even after a 40-min washout. Peak currents were plotted *versus* test potential and the minima, that is, maximum inward Na^+ currents, were used to calculate percent inhibition by anesthetics; for comparative purposes,[6,27] data were fit to rectangular hyperbolae (figs. 6C and D). F3 inhibited peak Na^+ current by 70 ± 9% at 0.6 mM (~0.6 MAC) with an IC_{50} of 0.24 ± 0.06 mM (n = 11); F6 inhibited by 16 ± 2% at 0.018 mM (~predicted MAC) with an IC_{50} of 0.020 ± 0.014 mM (n = 7). The voltage dependence of Na^+ current activation was not affected by either cyclobutane. Representative traces from two cells are shown in figure 7.

Volatile anesthetics have been shown to alter the voltage dependence of Na^+ channel steady state inactivation,[6] a physiologically important property of Na^+ channels that determines channel availability at specific membrane potentials.[28] The voltage protocol used to measure steady state inactivation consisted of prepulses to varying potentials (500 ms to potentials between −150 and −10 mV) and a constant test pulse (to +10 mV). Before cyclobutane application, Na^+ currents decreased at prepulse potentials more positive than −80 mV, because of a voltage-dependent transition from closed to inactivated states, the latter being unavailable for opening (fig. 8). F3 produced a reversible, concentration-dependent hyperpolarizing shift in steady state inactivation (fig. 8A). At 0.6 mM F3 (<1 MAC), steady state inactivation shifted −21 ± 9.3 mV (n = 4). At −60 mV (close to resting membrane potential), the combination of voltage-independent block and the shift in inactivation resulted in 55 ± 13% block (n = 7) of Na^+

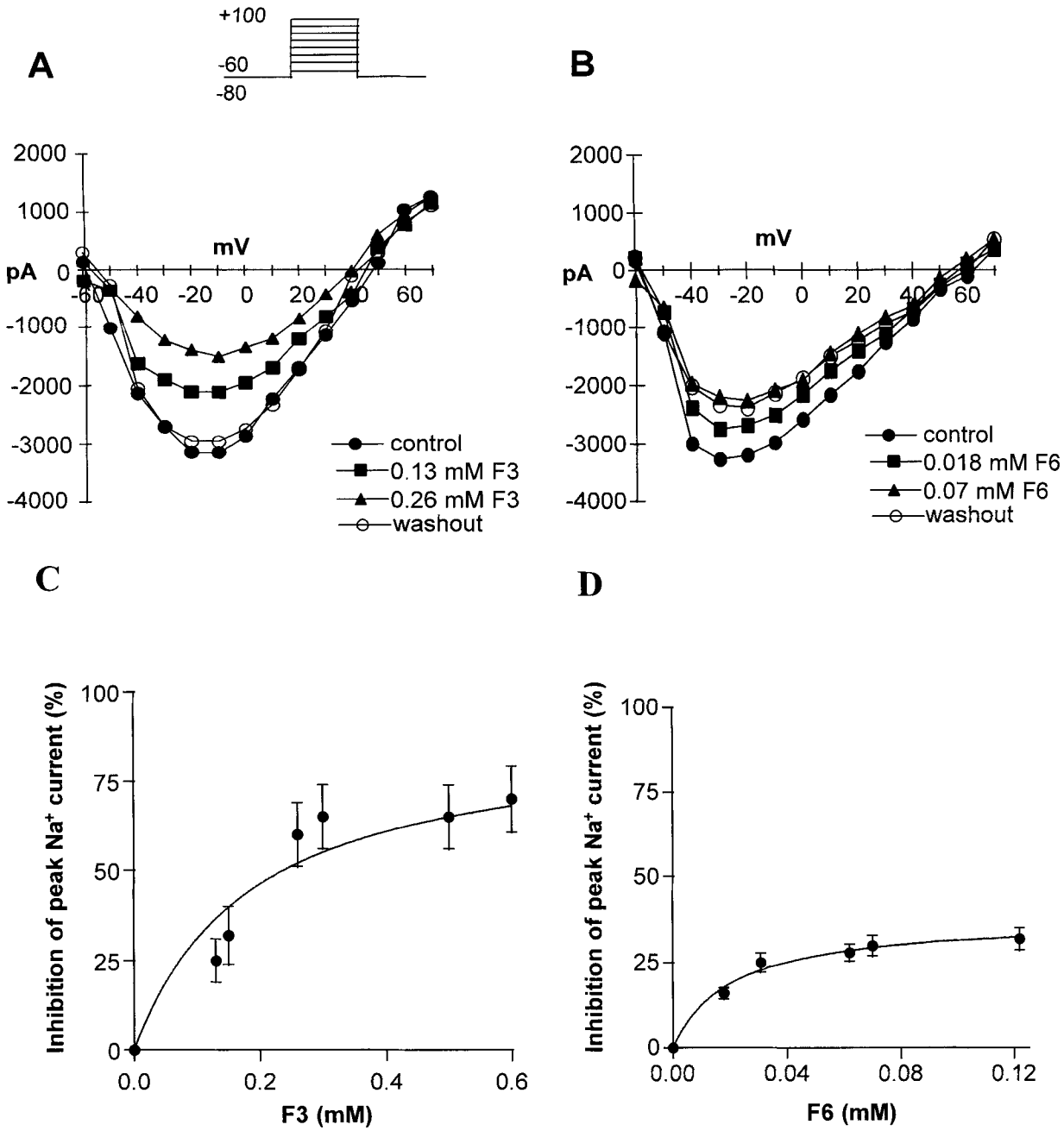

Fig. 6. Effects of F3 (*A, C*) and F6 (*B, D*) on whole-cell Na^+ current in isolated dorsal root ganglion neurons. (*A, B*) Current traces in the presence and absence of F3 and F6, respectively. Representative peak current–voltage relations are shown for these currents, elicited by voltage steps from a holding potential of −120 mV to test potentials varying from −60 to +100 mV in two separate cells. Washout traces were recorded after 20 min perfusion with buffer containing no cyclobutane. (*C, D*) Concentration–effect curves of pooled data for peak Na^+ current inhibition by F3 (*C*; n = 11) or F6 (*D*; n = 7), calculated as the percent inhibition of maximum inward current obtained from current–voltage plots from a holding potential of −100 mV. The maximal block of peak Na^+ currents by F3 was 70% at 0.6 mM (IC_{50} = 0.24 mM) and by F6 was 32% at 0.12 mM (IC_{50} = 0.02 mM).

current by 0.26 mM F3 and 67 ± 4% block by 0.6 mM F3 (n = 4). F6 had minimal effects on Na^+ channel steady state inactivation compared with F3 (fig. 8B), even at concentrations up to six times the predicted MAC. The average shift was −3.2 ± 0.9 mV (n = 5). The Na^+ current block at −60 mV had little dose-depen-

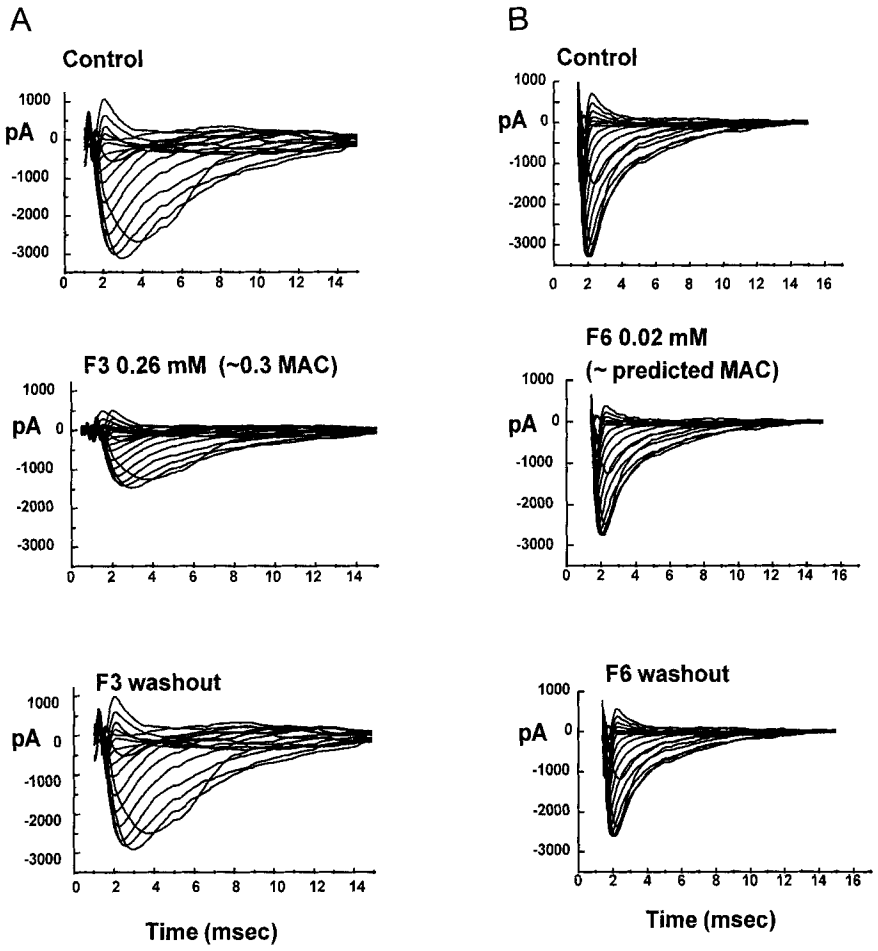

Fig. 7. Effects of F3 and F6 on whole-cell Na^+ current in isolated dorsal root ganglion neurons. Representative raw data for a single cell exposed to F3 (*A*) or F6 (*B*) are shown.

dence, ranging from 21% at 0.018 mM F6 to 28% at 0.12 mM F6.

In the F3 experiments (with up to 0.6 mM F3), full recovery (to 95–100% of control values) was obtained within 10–12 min of washout in five cells. In eight other cells, partial recovery was observed, but washout was not always performed to completion for technical reasons. It is not always possible to obtain full recovery of control values after washout in each experiment, even using conventional anesthetics.[6] In contrast, with F6, no recovery (not even partial) was observed with washout times up to 40 min (n = 5).

Discussion

Considerable evidence indicates that general anesthetics inhibit mammalian Na^+ channels at clinical concentrations.[2–5,7,16,29] We probed the role of Na^+ channels as targets for general anesthetic action using two structurally similar polyhalogenated cyclobutanes, both predicted to be anesthetics by their hydrophobicity. The anesthetic F3 inhibited Na^+ channel function in both synaptosomes and DRG neurons; the nonanesthetic F6 had no significant effects on synaptosomal Na^+ channels and reduced effects on Na^+ currents in DRG neurons at concentrations predicted to produce anesthesia.

Inhibition by F3 of Na^+ channel-dependent synaptosomal glutamate release and increases in $[Ca^{2+}]_i$ are similar to effects reported for conventional volatile anesthetics.[7,24] At ~1.5 MAC, halothane inhibited veratridine- and 4AP-evoked glutamate release by 50% in rat cortical synaptosomes.[7,24] Similar to F3, higher concentrations of halothane (0.9 mM [~3 times MAC]) also inhibited KCl-evoked increases in $[Ca^{2+}]_i$ in rat cortical synaptosomes.[24] The only difference between F3 and halothane was in the effects on KCl-evoked release. Although halothane had no significant effect on KCl-evoked glutamate release up to 0.9 mM (~three times

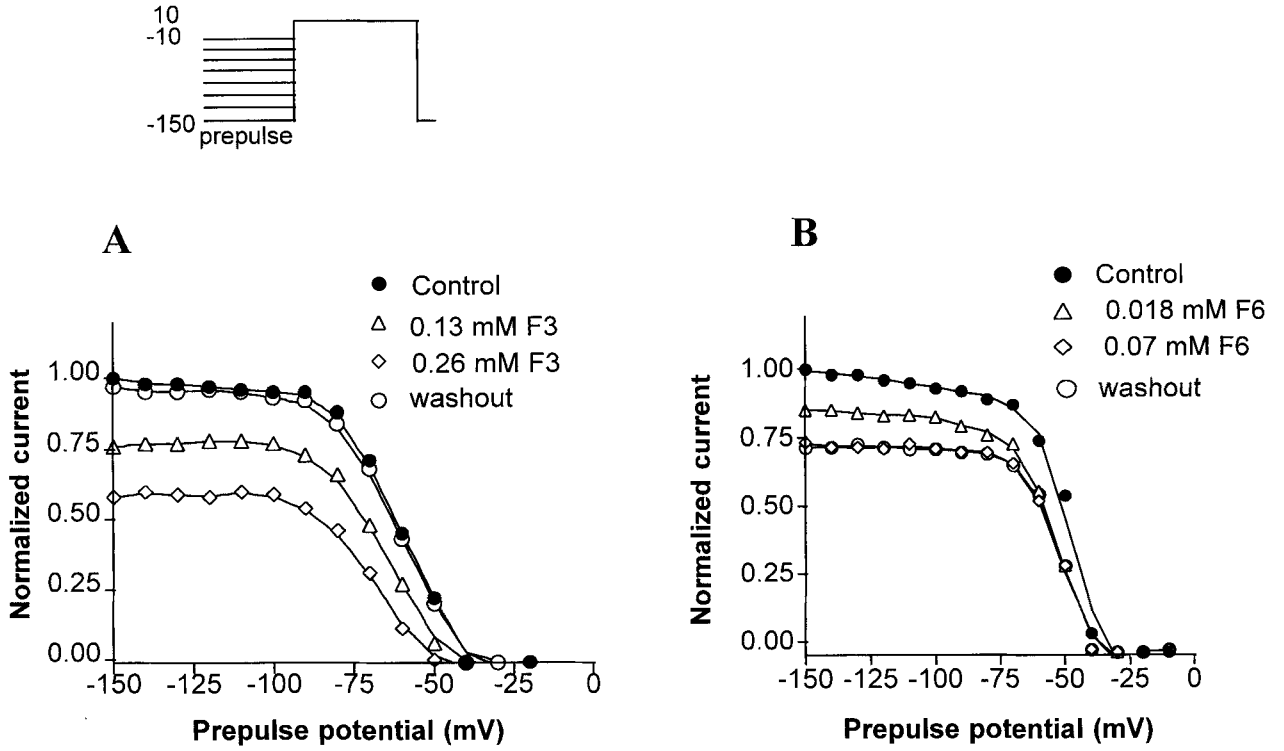

Fig. 8. Effects of F3 and F6 on Na^+ channel steady state inactivation. Na^+ currents were elicited by test pulses to −10 mV after 500-ms prepulses to potentials varying from −150 to −10 mV. Representative peak currents were normalized and plotted as a function of prepulse potential. F3 shifted inactivation by −21 mV at 0.6 mM (n = 4); F6 caused a shift of −3.2 mV at 0.07 mM (n = 5).

MAC[24]; Ratnakumari and Hemmings, unpublished observations), F3 (~three times MAC) inhibited KCl-evoked release by 69%. F3 at 1.6 mM has been reported to inhibit KCl-evoked glutamate release from mouse cerebrocortical slices.[30] In contrast to the results of Schlame and Hemmings,[24] Miao *et al.*[31] found significant inhibition of KCl-evoked glutamate release by clinical concentrations of isoflurane, halothane, and enflurane in guinea pig cortical synaptosomes. However, we found that isoflurane (up to 1 mM, ~three times MAC) inhibits 4AP-evoked glutamate release without affecting KCl-evoked release from cortical synaptosomes prepared from rats, mice, or guinea pigs (unpublished observations), ruling out a species difference as the explanation for this discrepancy.

In contrast to F3, F6 did not inhibit Na^+ channel-dependent glutamate release. Interestingly, F6 increased KCl-evoked glutamate release at 0.025 mM, but not at 0.05 mM, an effect that might contribute to its convulsant properties. A similar stimulatory effect by F6 on KCl-evoked glutamate release was reported in rat cortical brain slices.[32] However, a higher concentration of F6 (0.12 mM, five times predicted MAC), which is not achievable in cuvette methods for analyzing glutamate release and $[Ca^{2+}]_i$, inhibited KCl-evoked glutamate release in superfused mouse cerebrocortical slices.[30] Lower concentrations of F6 and the mechanism of the effect were not investigated in that study; therefore, it is unclear whether this was a direct effect of F6 on glutamate release. Nevertheless, clear differences between the effects of F3 and F6 were apparent in our study at concentrations relevant to anesthesia: F6 (0.05 mM, ~two times predicted MAC) had no effect on 4AP- or veratridine-evoked glutamate release or veratridine-evoked increases in $[Ca^{2+}]_i$; F3 at concentrations less than 1 MAC significantly inhibited these effects.

F3 was at least threefold more potent in inhibiting 4AP- or veratridine-evoked than KCl-evoked glutamate release. This is consistent with a more potent inhibition of Na^+ channels compared with Ca^{2+} channels coupled to glutamate release. The effects of F3 on veratridine- and KCl-induced increases in $[Ca^{2+}]_i$ support this observation. Less marked inhibition of KCl-induced (Na^+ channel-independent) increases compared with 4AP- or veratridine-evoked (Na^+ channel-dependent) increases in $[Ca^{2+}]_i$ or glutamate release has been observed for halo-

thane as well.[24] Increases in resting $[Ca^{2+}]_i$ observed in the presence of higher concentrations of F3 may result from increased synaptosomal membrane permeability to Ca^{2+}. Similar increases in resting $[Ca^{2+}]_i$ were observed with halothane and isoflurane in rat cerebrocortical synaptosomes[25] and in dissociated CA1 neurons and rat hippocampal slices.[33]

Inhibition of 4AP- and veratridine-evoked glutamate release by F3 could involve one or more of the following mechanisms: blockade of presynaptic Na^+ channels necessary for terminal depolarization; blockade of specific presynaptic Ca^{2+} channel subtypes coupled to glutamate release; interference with synaptic vesicle fusion and release mechanisms (*e.g.*, by affecting synaptic core complex function); inhibition of reversed Na^+-glutamate transport; and stimulation of glutamate reuptake. Our results with F3 and halothane suggest that inhibition of glutamate release by volatile anesthetics involves primarily blockade of Na^+ channels, although some contribution by the other mechanisms may also be involved, especially at higher concentrations at which blockade of Ca^{2+} channels is evident.

The effects of F3 and F6 on Na^+ currents in isolated DRG neurons were evaluated by whole-cell patch-clamp recording. These studies revealed two distinct effects of F3 on Na^+ currents: a potential independent suppression of resting or open Na^+ channels (for example, at hyperpolarized potentials at which inactivation is removed) and a hyperpolarizing shift in the voltage-dependence of Na^+ channel inactivation. This shift results in greater Na^+ channel inhibition by F3 at potentials close to normal neuronal resting membrane potentials compared with hyperpolarized potentials. In comparison, isoflurane (0.5 mM) caused a shift of −16 mV in the midpoint of channel inactivation in DRG neurons during similar conditions.[27] A similar voltage-independent block and hyperpolarizing shift in the steady state inactivation of rat CNaIIa Na^+ channel α subunits expressed in Chinese hamster ovary (CHO) cells occurs with halothane, isoflurane, enflurane, desflurane, sevoflurane, and ether.[6] The potency of F3 for inhibition of DRG neuron Na^+ currents (IC_{50} = 0.24 mM) correlates well with that for inhibition of Na^+ channel-dependent glutamate release (IC_{50} = 0.4 mM). The voltage-independent block at hyperpolarized potentials differed from that reported for conventional volatile anesthetics using rat CNaIIa Na^+ channels expressed in Chinese hamster ovary cells,[6] in that peak current block by F3 never reached 100%. This was not caused by differences between these anesthetics, because maximal block of peak Na^+ currents by isoflurane was 54% in DRG neurons during the same experimental conditions.[27] DRG neurons express several Na^+ channel isoforms with distinct functional properties, even within the same cell.[34] The failure of anesthetics to completely block peak Na^+ current in DRG neurons may result from different sensitivities of Na^+ channel isoforms within each cell, with some Na^+ channel isoforms having little or no anesthetic sensitivity. Differential effects on tetrodotoxin-resistant and -sensitive Na^+ channels did not contribute to this incomplete blockade because only tetrodotoxin-sensitive currents were recorded.

In contrast to F3, F6 was much less effective in blocking peak Na^+ currents and had no significant effect on the voltage dependence of channel inactivation. These results suggest that F6 may cause slight and irreversible inhibition of the closed or open states of the channel but has almost no interaction with its inactivated state. Although F6 blocked approximately 20–25% of DRG Na^+ current, no functional effects were observed in the synaptosome studies; F3 exhibited comparable potencies in both systems. This difference may result from differences between central nervous system presynaptic and peripheral DRG neuron Na^+ channel properties or the inability of the reduced block observed in DRG neurons to prevent depolarization of synaptosomes, or it may reflect the importance of the effects on inactivation as an anesthetic mechanism.

In neonatal rat spinal cord slices, F3 reversibly depressed glutamatergic monosynaptic reflex response and enhanced muscimol-evoked dorsal root potentials (a depolarizing response to activation of γ-aminobutyric acid A [$GABA_A$] receptors); F6 potentiated the former effect without affecting the latter.[35] These authors suggested that depression of glutamate receptors may contribute to the anesthetic actions of F3 and conventional volatile anesthetics and that enhancement of glutamate receptors by F6 may contribute to its convulsant activity. Our findings suggest that presynaptic inhibition of glutamate release is an important mechanism for inhibition of glutamatergic transmission[24,31,36,37] by F3 and conventional volatile anesthetics and may be functionally more important than postsynaptic antagonism of glutamate receptors.[37] Furthermore, an increase in glutamate release in addition to or rather than direct potentiation of postsynaptic glutamate receptors may contribute to the convulsant action of F6. The convulsant barbiturate 5-[2-cyclohexylidene-ethyl]-5-ethyl barbituric acid (CHEB) has also been found to increase glutamate release from cortical synaptosomes.[38]

Differential effects of anesthetic and nonanesthetic cyclobutanes on various recombinant ligand-gated ion channels expressed in *Xenopus* oocytes have also been reported. F3, but not F6, potentiated GABA-induced chloride (Cl^-) currents with recombinant human $\alpha_1\beta_2$- or $\alpha_1\beta_2\gamma_2$S-$GABA_A$ receptors.[39] F3 potentiated glycine-induced Cl^- currents mediated by human homomeric α_1-glycine receptors; F6 had no effect.[40] F3 enhanced rat kainate ($GluR_6$) receptors; F6 had no effect.[41] F3 inhibited $mGluR_5$ metabotropic glutamate receptor-induced Ca^{2+}-dependent Cl^- currents without affecting $mGluR_1$ receptor-induced currents; F6 inhibited the function of both receptors.[42] Muscarinic (M_1) receptor-induced Ca^{2+}-dependent Cl^- currents and 5-hydroxytryptamine type 2A receptor-induced currents were inhibited by F3 and F6 both; however, GF 109203X, a protein kinase C inhibitor, abolished the inhibitory effects of F3 but not of F6, suggesting a protein kinase C-mediated mechanism for F3 but not for F6.[43,44] These studies suggest that these structurally similar compounds, both predicted by their lipid solubility to produce anesthesia *in vivo*, have distinct pharmacologic properties *in vitro* that may be useful in distinguishing targets relevant to the immobilizing properties of volatile anesthetics.

Desflurane and F6 both are able to suppress learning in a fear-potentiated startle paradigm in rats, despite the inability of F6 to suppress movement in response to noxious stimuli.[12] This suggests that anesthetics and F6 may share certain anatomic and molecular sites of action involved in amnesia (probably supraspinal) and analgesia but differ in other sites (probably spinal) involved in immobility.[45] In addition to lipophilic properties, full anesthetic effectiveness necessitates substantial hydrophilic properties associated with higher molecular dipole moments and preferential localization to the membrane-water interface.[46,47] In contrast, nonanesthetics are highly lipophilic, with low hydrophilicity and molecular dipole moments, and distribute to the hydrophobic hydrocarbon membrane core. Thus, anesthetics appear to produce immobility by interacting with sites that possess polar and nonpolar properties; nonanesthetics and anesthetics appear to produce amnesia, and nonanesthetics convulsions, by interacting with nonpolar sites.[45]

In conclusion, the anesthetic cyclobutane F3, similar to conventional volatile general anesthetics, inhibited Na^+ channel-dependent glutamate release and Na^+ currents at concentrations observed during anesthesia *in vivo*; the nonanesthetic cyclobutane F6 had quantitatively and qualitatively distinct effects at concentrations predicted to produce anesthesia. These differential effects further support neuronal Na^+ channels as targets for general anesthetics.[2-7] Presynaptic inhibition of glutamate release mediated by Na^+ channel blockade appears to be a pharmacologically relevant effect of volatile anesthetics in producing immobilization, perhaps by inhibiting the spinal nocifensive reflex.[48]

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