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Closing and Inactivation Potentiate the Cocaethylene Inhibition of Cardiac Sodium Channels by Distinct Mechanisms

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ABSTRACT

Cocaethylene, a metabolite of cocaine and alcohol, is a potent inhibitor of the cardiac ($Na_v1.5$) sodium channel heterologously expressed in *Xenopus laevis* oocytes. Cocaethylene produces minimal tonic block under resting conditions but causes a potent use-dependent inhibition during repetitive depolarization and a hyperpolarizing shift in the steady-state inactivation. The data are consistent with a state-dependent binding mechanism, which has high affinity for inactivated channels (K_I = 17 μM) and low affinity for resting channels (K_R = 185 μM). Mutations of the interdomain D3-D4 linker eliminated rapid inactivation and weakened the cocaethylene inhibition, consistent with an important role for fast inactivation in cocaethylene binding. A rapid component of cocaethylene inhibition was observed in a noninactivating mutant of Nav1.5 that was tightly linked to channel opening and displayed properties consistent with a pore blocking mechanism. Hyperpolarization caused the noninactivating mutant channel to close, trapping cocaethylene and slowing the recovery. Mutation of a conserved isoleucine (I1756C) located near the extracellular end of the D4S6 segment accelerated the recovery of the noninactivating channel, suggesting that this mutation facilitates cocaethylene untrapping, which seems to be the rate-limiting step in the recovery when the channel is closed. This contrasts with the rapidly inactivating channel, where the I1756C mutation did not alter the recovery from cocaethylene inhibition. The data suggest that additional mechanisms, such as more stable cocaethylene binding, may be a more important determinant of recovery kinetics when the channels are inactivated. The data indicate that deactivation and inactivation slow the recovery and potentiate the cocaethylene inhibition of the Nav1.5 channel by distinct mechanisms.

Cocaine abuse is a major health concern in the United States, and its increased use has been paralleled by a rising incidence of cocaine-related cardiac arrhythmias and sudden death (Nanji and Filipenko, 1984; Isner and Chokshi, 1991). The majority of the people who use cocaine simultaneously consume alcohol (Weiss et al., 1988; Grant and Harford, 1990), which is believed to potentiate and prolong the euphoric effects of the drug (Farre et al., 1993). The combination of ethanol and cocaine produces greater increases in heart rate and blood pressure compared with either drug alone and increases the risk of sudden cardiac death (Knuepfer, 2003). Cocaethylene is a metabolite of cocaine and ethanol that is synthesized when the two drugs are coingested (Dean et al., 1991; Brzezinski et al., 1994). Cocaethylene slows cardiac conduction and delays repolarization, conditions that are known to promote arrhythmias (Erzouki et al., 1993; Wilson et al., 1995; Henning and Wilson, 1996). Cocaethylene is a potent inhibitor of the native cardiac Na^+ current (Xu et al., 1994). Rapid binding of cocaethylene to open and inactivated channels and slow dissociation from inactivated channels has been suggested to underlie the potent cocaethylene inhibition of the cardiac Na^+ channel (Xu et al., 1994). In addition, cocaethylene inhibits human *ether-a-go-go* potassium channels that produce the rapidly activating component of the cardiac delayed rectifier current (Ferreira et al., 2001; O'Leary, 2002). Cocaethylene inhibition of cardiac sodium and potassium channels may contribute to the increased incidence of sudden death associated with the combined use of cocaine and alcohol (Wilson et al., 1995; Henning and Wilson, 1996).

The anesthetic binding site of Na^+ channels has been localized to the cytoplasmic side of the channel and seems to be bounded by the selectivity filter on the external side and the channel gates on the internal side (Hille, 1992). Residues of the S6 segments of homologous domains (D) D1, D3, and D4 seem to line the internal vestibule and contribute to the cytoplasmic binding site (Ragsdale et al., 1994; Nau et al., 1999; Yarov-Yarovoy et al., 2001). Drugs access the anesthetic binding site by several distinct pathways. The best characterized is the internal aqueous pathway that permits charged forms of anesthetics to rapidly access the binding site from the cytoplasmic side of the channel. Depolarization causes Na^+ channels to open, facilitating drug access and converting the binding site to its high-affinity conformation. Access to the binding site is regulated by the activation gate,

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which prevents charged drugs from reaching the binding site under resting conditions and acts to trap drugs within the pore as the channels deactivate (Strichartz, 1973). A hydrophobic pathway has been proposed that permits the direct binding of uncharged drugs to the cytoplasmic binding site of closed and inactivated channels (Hille, 1977). Recent work indicates that permanently charged analogs of anesthetics may also gain access to the cytoplasmic binding site by permeating through the pore (Ragsdale et al., 1994; Qu et al., 1995; Sunami et al., 2000; Lee et al., 2001). The availability of these access pathways and the affinity of drug binding are believed to vary with the gating states of the channel and underlie the characteristic state-dependent inhibition produced by anesthetics and antiarrhythmic drugs (Hille, 1977; Hondeghem and Katzung, 1977; Starmer et al., 1984).

We have attempted to gain insight into the mechanism(s) linking cocaethylene binding to Na^+ channel gating by comparing the effects of this drug on the rapidly inactivating wild-type and a noninactivating mutant of the cardiac ($Na_v1.5$) Na^+ channel. In the wild-type channel, cocaethylene produces a characteristic use-dependent inhibition that is typical of drugs that preferentially bind to the open and/or inactivated states of the channel. The cocaethylene inhibition of the noninactivating mutant is more rapid than that of the wild type and displays properties that are consistent with a pore-blocking mechanism. Inactivation of the wild type and deactivation of the noninactivating mutant potentiate the cocaethylene inhibition and slow the recovery of the drug-modified channels at hyperpolarized voltages. The data suggest that closing and fast inactivation of the cardiac Na^+ channel are important determinants of the cocaethylene inhibition.

Materials and Methods

RNA Preparation and Oocyte Injection. The cDNA encoding the human cardiac ($Na_v1.5$) Na^+ channel (Gellens et al., 1992) in the pcDNA plasmid (Invitrogen) was linearized with XbaI and full-length capped mRNA-transcribed using the T7 promoter (mMessage mMachine; Ambion, Austin, TX). Oocytes were harvested from mature female *Xenopus laevis* (Xenopus I; Ann Arbor, MI). The animals were anesthetized by immersion in tricaine (1.5 mg/ml), and several ovarian lobes were surgically removed under sterile conditions. The adhering follicle cell layer was removed by incubating oocytes with 1 mg/ml collagenase (Sigma Chemical, St. Louis, MO) in calcium-free OR2 (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4) solution for 2 h. The oocytes were washed with calcium-free OR2 and transferred to OR3 (70% Leibovitz L-15 medium; Invitrogen, Carlsbad, CA) supplemented with 15 mM HEPES, pH 7.4, 5 mM L-glutamine, and 10 mg/ml gentamycin. Stage IV–V oocytes were microinjected with 50 nl of cRNA (1–2 $\mu g/\mu l$) and incubated for 24 to 48 h at 18°C.

Two-Electrode Voltage Clamp. The currents of cRNA-injected oocytes were recorded using a standard two-electrode voltage clamp technique. Oocytes are impaled with microelectrodes (<1 MΩ) filled with 3 M KCl and currents recorded using an OC-725C voltage clamp (Warner Instruments, Hamden, CT). Oocytes were held at −100 mV and pulses were generated using pClamp software (version 7; Axon Instruments, Foster City CA). Oocytes were incubated and currents recorded in frog Ringer solution containing 116 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4. All recordings were performed at room temperature (21–23°C).

Site-Directed Mutagenesis. Amino acid substitutions were made using the QuikChange site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA). Fragments (1–2 kb) encompassing the mutation sites were excised and subcloned into pcDNA (Invitrogen). Complementary pairs of mutagenic oligonucleotides (22–29 bp) containing the appropriate nucleotide substitutions were prepared at the Nucleic Acid Facility, Kimmel Cancer Center (Jefferson Medical College, Philadelphia, PA). These oligonucleotides were subsequently used as primers for the complete synthesis of both strands of the plasmid. We used 20 ng of cDNA plasmid as template, 5 U of *Pfu* DNA polymerase (Stratagene), primers, and free nucleotides in a total volume of 100 μl. After strand synthesis (≈ 20 cycles), 10 U of DpnI was added to the reaction mixture to digest the original methylated plasmid template (37°C, 1–2 h). The restriction endonuclease was heat-inactivated (65°C, 15 min), and the mixture was used to transform DH5α cells by electroporation. Base substitutions were confirmed by automated DNA sequencing by the Nucleic Acid Facility of the Kimmel Cancer Center. DNA fragments (1–2 kb) carrying the mutation were then subcloned into wild-type $Na_v1.5$ background and amplified in DH5α. The QQQ-I1756C and QQQ-Y1767C mutants were constructed by transferring the individual D4S6 mutations into the noninactivating (QQQ) mutant background. The QQQ-F1760C mutant was also constructed but failed to produce current when expressed in oocytes.

Data Analysis. Data are presented as the mean ± S.E.M. Statistical comparisons were made using a Student's t test with $p < 0.05$ considered to be a significant difference. Curve fitting and plotting was performed using Sigmaplot (SPSS Science, Chicago, IL). Cocaethylene was provided by the National Institute on Drug Abuse (Bethesda, MD).

Results

Frequency-Dependent Inhibition of $Na_v1.5$. A hallmark of many local anesthetics is a frequency or use-dependent inhibition of Na^+ channels that is observed during repetitive stimulation. This type of inhibition is characterized by a progressive decrease in the amplitude of the current elicited by rapidly applied depolarizing pulses and is believed to primarily reflect the incomplete recovery of drug-modified channels during the short rest interval between depolarizations. At low concentrations (≤50 μM), cocaethylene produces little resting block ($I_{drug}/I_{control}$ = 0.95 ± 0.04, n = 10) when the channels are held at a hyperpolarized voltage and stimulated at a low frequency (0.1 Hz). Consistent with what has been previously observed for many other local anesthetics, cocaethylene seems to bind with low affinity to closed channels. Use-dependent inhibition of $Na_v1.5$ was induced by applying a series of 20-ms depolarizing pulses at a frequency of 5 Hz (Fig. 1). In the absence of drug, the current amplitude (I) remains constant over the course of 50 pulses (I_{50}/I_1 = 1.0), indicating that inactivated channels fully recover during the 180-ms rest interval between pulses. In the presence of cocaethylene (50 μM), the amplitude of the current progressively decreases for successive pulses within the stimulation train (I_{50}/I_1 = 0.49) because the channels enter into a drug-modified state and only partially recover between pulses. The combination of weak resting block and potent use-dependent inhibition is consistent with the proposal that cocaethylene preferentially binds to the open and/or inactivated states of the channel.

To further investigate the state-dependence of cocaethylene binding, we examined its effects on the channels at voltages where the channels are predominately inactivated. Anesthetics that bind with high affinity to inactivated channels typically cause a hyperpolarizing shift in the steady-state availability (Weidman, 1955; Courtney, 1975; Bean et

al., 1983). We examined the binding of cocaethylene (10–150 μM) to inactivated channels by applying 5-s prepulses to voltages between −120 and −65 mV. A standard test pulse was used to assess the channel current, which was normalized to that measured at −120 mV and plotted versus the prepulse voltage (Fig. 1B). Cocaethylene caused a concentration-dependent hyperpolarizing shift in the inactivation. The data were fitted with Boltzmann functions, and the relative shifts in the midpoints were plotted versus the cocaethylene concentration (Fig. 1C). The smooth curve is a fit to a modified form of the Modulated Receptor model (Fig. 1C, inset) with resting and inactivated state affinities of 185 and 17 μM, respectively (Bean et al., 1983).

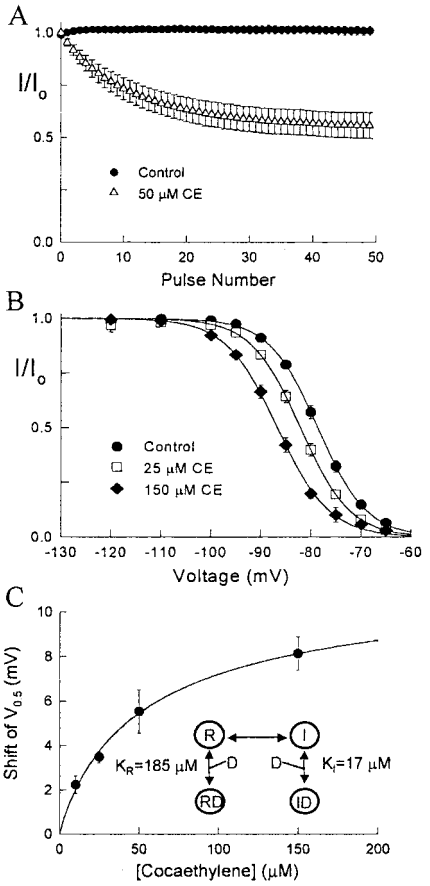

Fig. 1. Cocaethylene inhibition of $Na_v1.5$ channels expressed in *X. laevis* oocytes. $Na_v1.5$ Na^+ channels were expressed in oocytes and currents recorded using two-electrode voltage clamp. Use-dependent inhibition was induced by applying a series of 50 depolarizing pulses (−10 mV/20 ms, V_H = −100 mV) delivered at frequency of 5 Hz. The peak currents were normalized to the current elicited by the first pulse and plotted versus the pulse number. An estimate of the steady state cocaethylene inhibition (50 μM) was obtained from the ratio of currents elicited by the 50th and 1st pulses (I_{50}/I_1). The I_{50}/I_1 ratios for control and after application of 50 μM CE were 1.0 ± 0.02 and 0.49 ± 0.04, respectively. B, inactivation was induced by applying 5-s pulses to voltages between −120 and −65 mV. The steady-state availability was then assayed using a standard test pulse (−10 mV/20 ms). The test currents were normalized to the current measured from the −120 mV holding potential and plotted versus voltage. Cocaethylene (10–150 μM) was applied for 5 min before repeating the pulsing protocol. The smooth curves are fits of the data to the Boltzmann function [$h_\infty = 1/(1 + \exp(V - V_{0.5})/k)$] with midpoints ($V_{0.5}$) and slope factors ($k_v$) of −78.6 ± 0.06 mV and 4.8 ± 0.06 mV (Control), −80.8 ± 0.05 mV and 5.0 ± 0.05 mV (10 μM), −82.1 ± 0.15 and 4.9 ± 0.13 mV (25 μM), −84.1 ± 0.09 mV and 5.0 ± 0.07 (50 μM), and −86.7 ± 0.14 mV and 5.1 ± 0.13 mV (150 μM) (n = 5). C, the relative shifts ($\Delta V_{0.5}$) produced by cocaethylene were calculated from paired data [$(V_{0.5}(CE) - V_{0.5}(Cont)$] and plotted versus the cocaethylene concentration. Inset, modulated receptor model used to evaluate the resting (R) and inactivated (I) state affinity of cocaethylene binding. The smooth curves are fits to the modulated receptor equation [$\Delta V_{0.5} = k_v \ln((1 + [CE]/K_R)/(1 + [CE]/K_I))$] with K_R and K_I of 185 ± 55 μM and 16.9 ± 1.9 μM, respectively. k_v was fixed to 4.8 mV, reflecting the mean slope factor of the control data.

The D4S6 segment of Na^+ channels is known to play an important role in the binding of local anesthetics (Ragsdale et al., 1994). We replaced two of the highly conserved residues of the D4S6 segment previously shown to contribute to local anesthetic binding (Ile1756, Tyr1767) with cysteines and examined the effects of these mutations on the use-dependent inhibition (Fig. 2). In the presence of 50 μM cocaethylene, the peak current of the I1756C mutant was reduced 30.5 ± 1.7% (n = 5) after applying five depolarizing pulses versus 17.2 ± 0.9% (n = 6) for the wild-type channel. The I1756C mutation located near the extracellular end of the D4S6 segment slightly accelerated the onset but did not alter the steady-state inhibition (I_{50}/I_1 = 0.46) compared with the wild-type channel (I_{50}/I_1 = 0.49). The I1756C mutation seemed to facilitate drug binding to the internal binding site, consistent with what has been observed previously for similar mutations in neuronal and skeletal muscle Na^+ channels (Ragsdale et al., 1994; Sunami et al., 2001). This contrasts with mutations near the cytoplasmic end of the D4S6 (Y1767C), which significantly weakened the use-dependent inhibition (I_{50}/I_1 = 0.89), suggesting that this residue may directly contribute to cocaethylene binding (Fig. 2B).

Recovery from Cocaethylene Inhibition. The use-dependent inhibition produced by cocaethylene suggests that the drug-modified channels slowly recover at hyperpolarized voltages. We examined the effect of cocaethylene on the recovery time course using a triple-pulse protocol consisting of

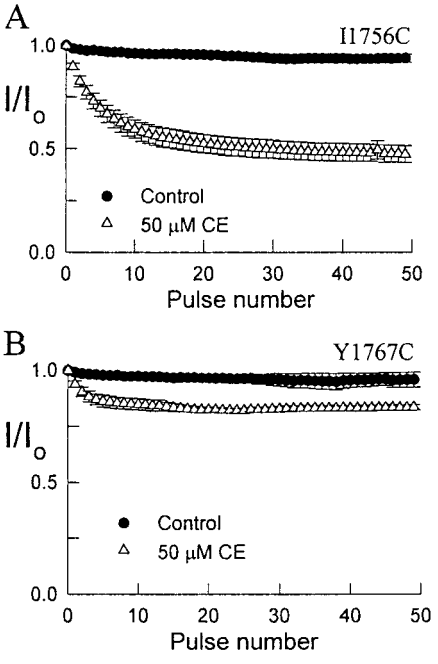

Fig. 2. Use-dependent inhibition of D4S6 mutants. The use-dependent inhibition of the D4S6 (I1756C, Y1767C) mutant $Na_v1.5$ channels was determined as described in Fig. 1A. The peak currents were normalized to the current elicited by the first pulse and plotted versus the pulse number. The I_{50}/I_1 ratios for control and after application of 50 μM CE were 0.94 ± 0.02 and 0.46 ± 0.03 for I1756C (n = 7), 0.97 ± 0.01 and 0.89 ± 0.02 for Y1767C (n = 7).

a prolonged depolarization (−10 mV/10 s) to inactivate the channels, a variable duration recovery at −100 mV (1 ms–30 s) and standard test pulse to assay availability (Fig. 3A). In the absence of cocaethylene, the recovery is best fit by the sum of three exponentials with time constants of 8, 140, and 1440 ms (Table 1). Cocaethylene slows the recovery time course, selectively increasing the time constants of the intermediate (τ = 310 ms) and slow components (τ = 4610 ms). Although the relative amplitude of the fast component was reduced by 65% in the presence of cocaethylene, the recovery time constant (τ = 5 ms) was not different from the control (τ = 8 ms) and is therefore likely to reflect the recovery of drug-free channels. During the depolarizing prepulses, the $Na_v1.5$ channels enter into a combination of fast and slow inactivated states. Cocaethylene seems to slow the recovery of the channels at hyperpolarized voltages by stabilizing the channels in one or more of these inactivated states.

The effects of the D4S6 mutations on the time course of repriming were also investigated (Fig. 3, C and D). In the absence of drug, the recovery of the D4S6 mutants was not substantially different from the wild-type channel. Cocaethylene slows the recovery of the I1756C mutant by selectively increasing the time constants of the intermediate and slow components similar to that observed for the wild type (Table 1). By contrast, the Y1767C mutation substantially attenuated the effect of cocaethylene on the recovery. The difference in the recovery time course of the control and drug-modified Y1767C mutant is attributed to a 40% reduction in the fraction of channels recovering with the fast time constant and a concomitant increase in the relative amplitudes of the intermediate and slow components (Table 1). Although the shift in the relative amplitude toward the slower recovering components is consistent with substantial cocaethylene binding, the recovery of the mutant channel is more rapid than the wild type. The data suggest that the Y1767C mutation causes cocaethylene to rapidly dissociate at hyperpolarized voltages. The more rapid recovery of the drug-modified Y1767C channel may explain the relatively weak use-dependent inhibition of this mutant channel (Fig. 2B).

Time Course of Cocaethylene Binding to Inactivated Channels. The use-dependent inhibition produced by cocaethylene suggests that conformational changes associated with the opening or inactivation of the channel contribute to the binding of this drug. Unfortunately, rapid repetitive pulsing causes the channels to cycle through activated and inactivated conformations, making it difficult to unequivocally identify the states important for cocaethylene binding. To further investigate the state-dependent inhibition produced by cocaethylene, variable-duration depolarizing voltage pulses (1 ms–30 s) were applied to promote and stabilize the channels in inactivated states (Fig. 4A). A short hyperpolarization (−150 ms) was used to permit the recovery of fast inactivated (τ = 8 ms), but not slow inactivated (τ = 1440 ms) or drug-modified channels (τ = 4610 ms). A standard test pulse (−10 mV/20 ms) was then used to assay the availability of the channels. The amplitudes of the test currents were normalized to control currents measured after a prolonged rest at −100 mV (>60 s) and plotted versus the prepulse duration (Fig. 4B). In the absence of drug, the current elicited by test pulses progressively decreases with the prepulse duration. The decay of the current was fitted by a single exponential with a constant (τ) of 4.8 s (A = 0.36), reflecting the slow inactivation of the channels. Cocaethylene (50 μM) accelerated the time course of the current decay (τ = 1.1s) and increased its relative amplitude (A = 0.65), consistent with the cocaethylene inhibition of the channels during the depolarizing prepulse. Although the channels open during the depolarizing prepulses, they do so briefly near the beginning of the pulse and at −10 mV rapidly enter into absorbing inactivated states (τ = 0.7 ± 0.03 ms, n = 6) from which they

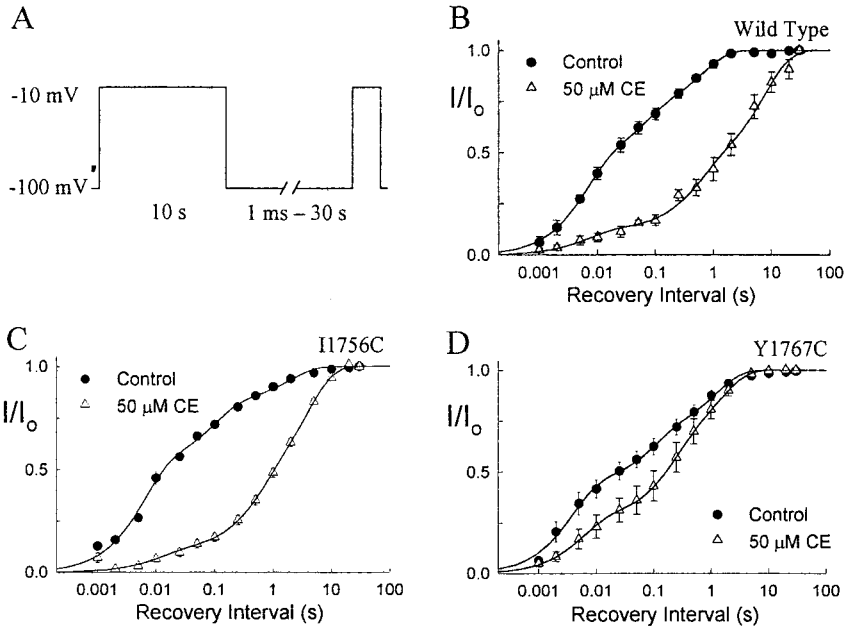

Fig. 3. Recovery from cocaethylene inhibition. A, the time course of recovery from inactivation and cocaine inhibition was measured using a triple-pulse protocol. B, the recovery of the wild type channels before (●, n = 4) and after application (△, n = 4) of 50 μM cocaethylene. The smooth curves are best fits to the sum of three exponentials [$I/I_o = 1 - (A_1 \times \exp(-t/\tau_1) + A_2 \times \exp(-t/\tau_2) + A_3 \times \exp(-t/\tau_3))$], where τ_1 to τ_3 are the recovery time constants and A_1 to A_3 are the corresponding relative amplitudes. The fitted parameters are listed in Table 1. C and D, recovery of the D4S6 mutants before (I1756C, n = 6; Y1767C, n = 5) and after (I1756C, n = 13; Y1767C, n = 7) application of 50 μM cocaethylene.

do not readily recover. The majority of the cocaethylene inhibition produced by these long depolarizations primarily reflects the high-affinity binding of cocaethylene to inactivated channels.

Unfortunately, our pulsing protocol does not distinguish between cocaethylene binding to fast and slow inactivated channels, both of which are believed to contribute to anesthetic binding (Hille, 1977; Hondeghem and Katzung, 1977; Chen et al., 2000). To further investigate the specific role of fast inactivation in cocaethylene binding, we examined the inhibition of a noninactivating mutant of $Na_v1.5$. The noninactivating mutant was constructed by replacing a trio of hydrophobic residues of the linker between homologous domains D3 and D4 with glutamines (IFM→QQQ) (West et al., 1992). Studying the cocaethylene inhibition of this mutant channel is useful because the QQQ substitutions are predicted to selectively disrupt components of drug binding associated with fast inactivation. We used our standard double pulse protocol (Fig. 4A) to measure the development of slow inactivation and cocaethylene inhibition in the QQQ mutant. In the absence of drug, the slow inactivation of the QQQ mutant (Fig. 4C) was more complete than that observed in the wild-type channel (Fig. 4B). This is consistent with previous studies showing that eliminating rapid inactivation potentiates the slow inactivation of the $Na_v1.5$ channel (Richmond et al., 1998). In the absence of drug, the decay of the QQQ mutant current was biexponential, with time constants of 61 ms (A = 0.07) and 6.3 s (A = 0.87). Application of cocaethylene (50 μM) accelerated the decay of current, which was also biphasic with fast and slow time constants of 36 ms (A = 0.31) and 3.3 s (A = 0.62) respectively. Cocaethylene induces a rapid component of current inhibition in the QQQ mutant (τ = 36 ms) that is considerably faster than that observed in the wild-type channel (τ = 1.1 s). In the next section, we will show data indicating that the persistent cocaethylene inhibition of the QQQ mutant displays properties that are consistent with a pore-blocking mechanism. The slow component of cocaethylene inhibition observed in the

TABLE 1
Time constants of recovery from inactivation
The time constants (τ_1–τ_3) and the relative amplitudes (A_1–A_3) obtained from curve fitting the recovery of the rapidly inactivating channels (Fig. 3).

	τ_1	τ_2	τ_3	A_1	A_2	A_3
	s	*s*	*s*			
Wild Type						
Control	0.008 ± 0.002	0.14 ± 0.06	1.44 ± 0.36	0.43 ± 0.04	0.26 ± 0.05	0.30 ± 0.06
50 μM CE	0.005 ± 0.002	0.31 ± 0.08	4.61 ± 0.35	0.14 ± 0.02	0.23 ± 0.03	0.62 ± 0.03
I1756C						
Control	0.006 ± 0.001	0.092 ± 0.04	1.86 ± 0.76	0.51 ± 0.06	0.30 ± 0.06	0.19 ± 0.04
50 μM CE	0.012 ± 0.009	0.54 ± 0.23	4.02 ± 0.066	0.10 ± 0.03	0.30 ± 0.08	0.60 ± 0.09
Y1767C						
Control	0.004 ± 0.001	0.09 ± 0.04	1.21 ± 0.28	0.44 ± 0.04	0.25 ± 0.05	0.31 ± 0.05
50 μM CE	0.006 ± 0.001	0.22 ± 0.02	1.53 ± 0.10	0.27 ± 0.01	0.36 ± 0.02	0.37 ± 0.02

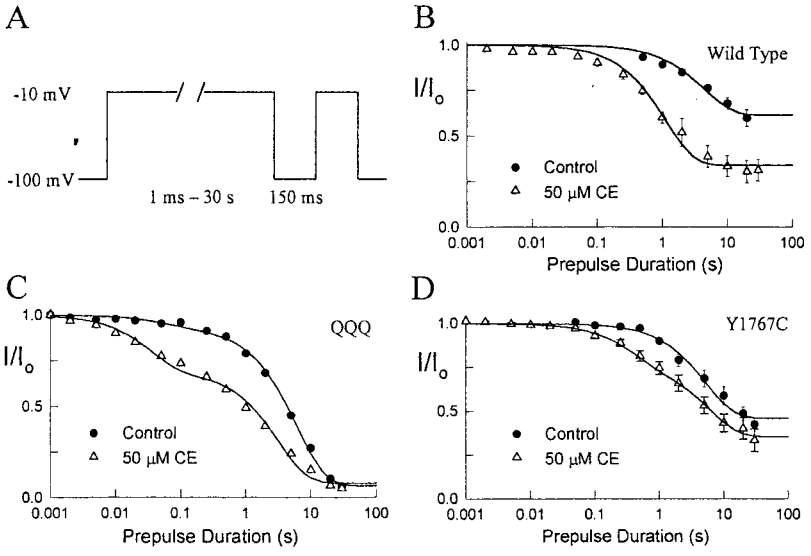

Fig. 4. Time course of cocaethylene inhibition. A, the development of cocaethylene (50 μM) inhibition at −10 mV was measured using a triple-pulse protocol. The currents elicited by the test pulses were normalized (I/I_o) to control currents measured after a prolong rest (>60 s) at −100 mV and plotted versus the prepulse duration (t). B, onset of cocaethylene inhibition of the wild-type channel. The time course of the current decay was fitted to an exponential function ($I/I_o = A \times \exp(-t/\tau)+A\infty$), where τ is the time constant, A the relative amplitude, and A∞ the residual current amplitude. For the control, the time constants before and after application of 50 μM cocaethylene were 4.8 ± 1.1 s (A = 0.36 ± 0.04, n = 6) and 1.1 ± 0.1 s (A = 0.65 ± 0.03, n = 12), respectively. C, development of the cocaethylene inhibition of the noninactivating QQQ mutant (see text). In the absence of drug, the time course of the current decay was fitted with the sum of two exponentials with time constants of 61 ± 45 ms (A = 0.07 ± 0.02) and 6.3 ± 0.4 s (A = 0.87 ± 0.02, n = 12) before and 36 ± 10 ms (A = 0.31 ± 0.03) and 3.3 ± 0.5 s (A = 0.62 ± 0.03, n = 12) after application of 50 μM cocaethylene. D, development of cocaethylene inhibition in the Y1767C mutant. In the absence of drug, the decay of the Y1767C control current was exponential with a time constant of 4.8 ± 1.1 s (A = 0.59 ± 0.05, n = 4). After application of cocaethylene the onset of the inhibition was biexponential with time constants of 0.3 ± 0.1 s (A = 0.29 ± 0.02) and 6.8 ± 0.8 s (A = 0.50 ± 0.02), respectively (n = 8).

QQQ mutant (τ = 3.3 s) is approximately 2-fold faster than the development of the slow inactivation in the drug-free control (τ = 6.3 s). The mechanism underlying this slow component of cocaethylene inhibition is not known, but may reflect drug interaction with inactivated channels. Overall, the data indicate that the QQQ substitutions accelerated and substantially weakened the cocaethylene inhibition, suggesting that conformational changes associated with fast inactivation significantly contribute to cocaethylene binding.

The Y1767C mutation reduced the use-dependent inhibition produced by cocaethylene (Fig. 2C) and accelerated the recovery of drug-modified channels (Fig. 4D), suggesting that this residue may contribute to cocaethylene binding. We further examined the effect of Y1767C mutation on cocaethylene binding to inactivated channels (Fig. 4D). In the absence of drug, the Y1767C mutant inactivates with a time constant of 4.6 s, similar to what is observed for the slow inactivation of the wild-type channel (τ = 4.8 s). In the presence of cocaethylene, the time course of the current decay was best fitted by the sum of two exponentials with fast and slow time constants of 0.4 and 6.2 s, respectively. The slower time constant is similar to that observed for the drug-free controls, suggesting that a substantial fraction of the Y1767C mutant channels (42%) inactivate normally and do not bind the drug. The fast component accounts for 23% of the current decay and may reflect a more rapid onset of the cocaethylene inhibition. The combination of rapid kinetics and reduced steady-state inhibition further supports the idea that the Y1767C mutation weakens the cocaethylene inhibition by causing the drug to rapidly dissociate from the binding site.

Cocaethylene Inhibition of the Noninactivating Mutant Channel. We further investigated the mechanism of cocaethylene inhibition of the QQQ mutant by applying long depolarizing pulses to directly measure the onset of the inhibition. The QQQ mutant rapidly activates but only slowly inactivates during prolonged (2 s) depolarizations (Fig. 5). In the absence of drug, the decay of the current was fitted by the sum of two exponentials with time constants of 126 ± 22 ms (A_1 = 0.06) and 1622 ± 57 ms (A_2 = 0.52, n = 5). This current decay seems to reflect the slow inactivation of the channel. Cocaethylene (50 μM) accelerates the time course of the current decay, which was fitted with the sum of three exponentials with time constants of 21 ± 1 ms (A_f = 0.17), 250 ± 15 ms (A_1 = 0.14), and 1529 ± 82 ms (A_2 = 0.44, n = 6). Cocaethylene induces a new rapid component of current decay that displays properties reminiscent of a simple pore blocking mechanism (Bennett et al., 1995; Pugsley and Goldin, 1999; O'Leary and Chahine, 2002).

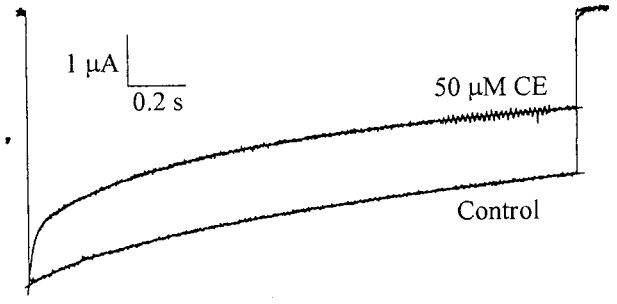

Fig. 5. Cocaethylene inhibition of the noninactivating (QQQ) mutant channel. Current was elicited by applying a 2-s depolarization to −10 mV from a holding potential of −100 mV. In the absence of drug, the decay of the current was biexponential with time constants of 161 and 1835 ms. After application of 50 μM cocaethylene, the decay was fitted with the sum of three exponentials with time constants of 19, 284, and 1485 ms.

Increasing the concentration of cocaethylene (25–250 μM) accelerated the time course of the QQQ current decay but only slightly reduced the peak current amplitude (Fig. 6A). An estimate of the resting block was obtained by extrapolating the time course of the current decay back to the beginning of the voltage pulse. These values were then normalized to the amplitude of the control (i.e., drug-free) current. In the presence of 250 μM cocaethylene, the relatively peak amplitude was 0.75 ± 0.06 versus the extrapolated current amplitude of 1.0 ± 0.07 (n = 3). Over the range of cocaethylene concentrations investigated, the majority of the inhibition occurs after the channels have opened with relatively little resting block of the QQQ mutant channels. The concentration-dependent increase in the onset of the cocaethylene inhibition is consistent with a simple pore blocking mechanism (Bennett et al., 1995; Grant et al., 1996; Pugsley and Goldin, 1999; Kimbrough and Gingrich, 2000; O'Leary and Chahine, 2002). The decay of the current measured during the initial 200 ms of depolarization was fitted with the sum of two exponentials with the fast component (τ_f) reflecting the rapid cocaethylene block. The linear relationship between the apparent blocking rate ($1/\tau_f$) and cocaethylene concentration (Fig. 6D) is consistent with a simple bimolecular interaction (O'Leary and Chahine, 2002). The association (k_{on}) and dissociation (k_{off}) rate constants are 4.73 × 10^5 M^{-1} s^{-1} and 28.4 s^{-1}, respectively, yielding an equilibrium constant (K_D = k_{off}/k_{on}) for cocaethylene binding of 59 μM.

We investigated the contribution of the D4S6 segment to the cocaethylene block of open channels by transferring the I1756C and Y1767C mutations to the noninactivating QQQ mutant background. Cocaethylene inhibited the QQQ-I1756C mutant in a concentration-dependent fashion (Fig. 6B). The blocking affinity of the QQQ-I1756C mutant (K_D = 44 μM) is slightly higher than what was observed for the QQQ control (K_D = 59 μM), which seems to result from a 2-fold increase in the association rate constant (k_{on} = 7.94 × 10^5 M^{-1} s^{-1}). The I1756C mutation seems to facilitate cocaethylene access to the cytoplasmic binding site, which may explain the more rapid onset of the use-dependent inhibition observed with this mutation (Fig. 2B). By contrast, the cocaethylene inhibition of the QQQ-Y1767C mutant is considerably weaker than the QQQ control (K_D = 481 μM), an effect that can be attributed to a 10-fold increase in the k_{off} (299.6 s^{-1}) with no change in k_{on} (4.77 × 10^5 M^{-1} s^{-1}). The Y1767C mutation weakens the block by causing cocaethylene to rapidly dissociate from the binding site, which may explain the reduced cocaethylene inhibition (Figs. 2B and 4D) and the rapid recovery of drug-modified channels at hyperpolarized voltages (Fig. 3D). These data are consistent with our previous observation that Tyr1767 contributes to a common anesthetic binding site for both the open and inactivated conformations of the $Na_v1.5$ channel (O'Leary and Chahine, 2002).

We also analyzed the steady-state cocaethylene inhibition of the QQQ mutant channels. The current amplitudes were measured near the end of the 400-ms depolarizing pulses (Fig. 6) and normalized to the drug-free controls. The data were fitted with a single-site binding model with apparent K_D of 97 ± 13 μM for QQQ (n = 6), 104 ± 26 μM for QQQ-I1756C (n = 4), and 319 ± 81 μM for QQQ-Y1767C (n =

5). Although the steady-state inhibition generally parallels the results of our kinetic analysis, the absolute K_D values obtained from the two methods are substantially different. We speculate that the steady-state determinations are highly sensitive to the resting block of the channels, which seems to be greater for the QQQ-I1756C mutant, and that cocaethylene binding may be modified by slow inactivation induced by prolonged depolarization. Additional studies of these parameters on cocaethylene binding are necessary to reconcile the discrepancy in the steady state and kinetic determinations of the binding affinity.

Cocaethylene Becomes Trapped as the Channels Close. Permanently charged analogs of anesthetics block Na^+ channels when open and become trapped within the pore as the channels deactivate at hyperpolarized voltages (Strichartz, 1973). These observations are consistent with an important role for the activation gate in the binding and unbinding of these drugs. The cocaethylene inhibition of the noninactivating QQQ mutant exhibits properties that are consistent with an open-channel blocking mechanism. We were interested in determining whether the cocaethylene block of the noninactivating mutant is stabilized at hyperpolarized voltages consistent with a trapping mechanism. The recovery of cocaethylene-modified channels was determined by first applying a depolarizing prepulse (−10 mV/100 ms) to open the QQQ mutant channels and to promote the cocaethylene block. The voltage was stepped to −100 mV for a variable duration (1 ms–30 s) before applying a standard test pulse to assay the fractional recovery (Fig. 7A). In the absence of drug, a small fraction (9%) of the channels inactivate during the depolarizing prepulse and recover with an apparent time constant of 0.7 s (Fig. 7B). This component of the recovery is likely to reflect channels entering into slow inactivated states during the depolarizing prepulse. In the presence of cocaethylene (100 μM), the initial reduction in the current amplitude increased 4-fold compared with the drug-free control and the current recovers with a time constant of 4.6 s. Channels that were blocked by cocaethylene during the depolarizing prepulse only slowly recover at the hyperpolarized voltage. This slow recovery cannot be attributed to cocaethylene binding to the fast-inactivated channels. Rather, these observations are in good agreement with the proposed pore-blocking mechanism and suggest that cocaethylene becomes trapped within the internal vestibule as the channels close at the hyperpolarized voltage. The untrapping from closed channels is slow, suggesting that this mechanism may contribute to the use-dependent inhibition (Fig. 1A) and the slow recovery of the wild-type channel (Fig. 4B).

To further investigate the underlying mechanism, we examined the voltage sensitivity of cocaethylene untrapping. A similar pulse protocol was used as in Fig. 7A except that the recovery voltage was varied between −120 and −80 mV. We observed only minor differences in the recovery kinetics indicating that cocaethylene untrapping is not voltage-dependent (Fig. 7C). This contrasts with the deactivation of $Na_v1.5$, which displays considerable voltage sensitivity between −120 and −80 mV (O'Leary and Horn, 1994). The data suggest that the voltage-dependent conformational changes associated with channel closing are not tightly linked to cocaethylene untrapping.

I1756C Facilitates Closed-State Untrapping. Previous studies indicate that mutations near the external end of the D4S6 segment facilitate the access of externally applied quaternary analogs of anesthetics to the cytoplasmic binding site of the Na^+ channel (Ragsdale et al., 1994; Qu et al., 1995; Lee et al., 2001; Sunami et al., 2001). Mutations within this region seem to create a pathway that enhances the permeation of these permanently charged drugs. The I1756C mutation, located near the external end of the D4S6 segment, facilitated cocaethylene binding to the open state of QQQ

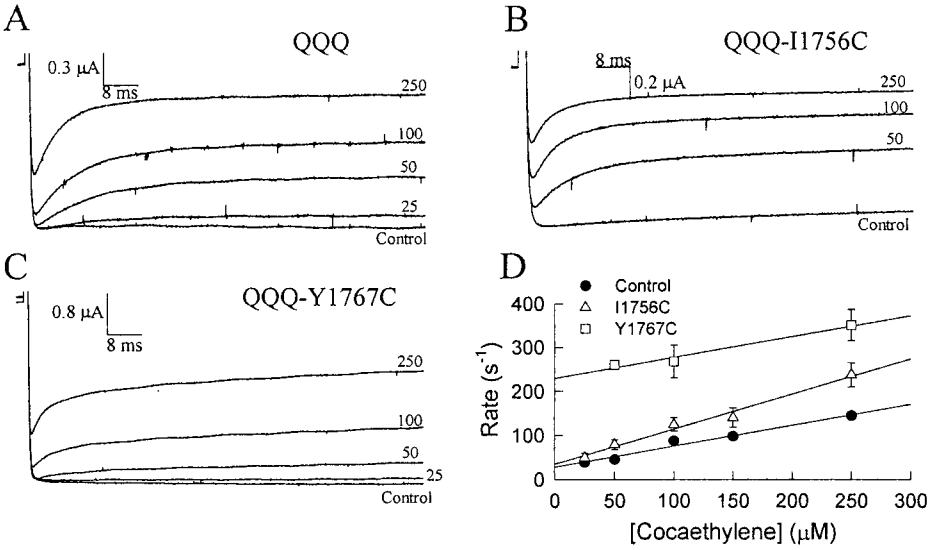

Fig. 6. Concentration-dependent inhibition of the noninactivating (QQQ) mutant channel. The time course of cocaethylene block was measured by depolarizing to −10 mV for 400 ms from a holding potential of −100 mV. A, cocaethylene (25–250 μM) induces a concentration-dependent decay in the current of the QQQ mutant channel. The current decay was fitted with the sum of two exponentials where the rapid component (τ_f) reflects the cocaethylene block. Also shown are the currents of the QQQ-I1756C (B) and QQQ-Y1767C mutants (C). D, plot of the blocking rate ($1/\tau_f$) versus cocaethylene concentration ([CE]) is linear ($1/\tau_B = k_{on}[CE] + k_{off}$) consistent with a simple bimolecular interaction where the slope is the association rate constant (k_{on}) and the Y-intercept the dissociation rate constant (k_{off}). The k_{on} and k_{off} were 4.7×10^5 M^{-1} s^{-1} and 28.4 s^{-1} for the QQQ; 7.9×10^5 M^{-1} s^{-1} and 35.0 s^{-1} for QQQ-I1756C; and 4.8×10^5 M^{-1} s^{-1} and 229.6 s^{-1} for QQQ-Y1767C. Data are the means of 12 (QQQ), 11 (QQQ-I1756C), and 9 (QQQ-Y1767C) individual experiments.

mutant channel (Fig. 6) and accelerated the onset of the use-dependent inhibition in the rapidly inactivating channel (Fig. 2). Consistent with previous studies, our data suggest that Ile1756 may be an important determinant of cocaethylene accessibility to the cytoplasmic binding site. To further test this mechanism, we examined the effect of the I1756C mutation on cocaethylene untrapping from the QQQ mutant. In the absence of cocaethylene, 12% of the QQQ-I1756C channels enter into the slowed inactivated state (Fig. 8A). Cocaethylene (100 μM) caused a 4-fold increase in the relative amplitude of slowly recovering component and increased the recovery time constant, consistent with cocaethylene block during the depolarizing prepulse and slow untrapping at −100 mV (Table 2). The untrapping of the QQQ-I1756C mutant (τ = 0.8 s) is considerably more rapid than that of the QQQ control (τ = 4.6 s). This faster recovery cannot be attributed to the rapid dissociation of the drug because the I1756C mutation increases the affinity of cocaethylene binding (Fig. 6D). Rather, the data indicate that the I1756C mutation facilitates the recovery of the drug-modified channel by permitting cocaethylene to rapidly escape when the channel is closed.

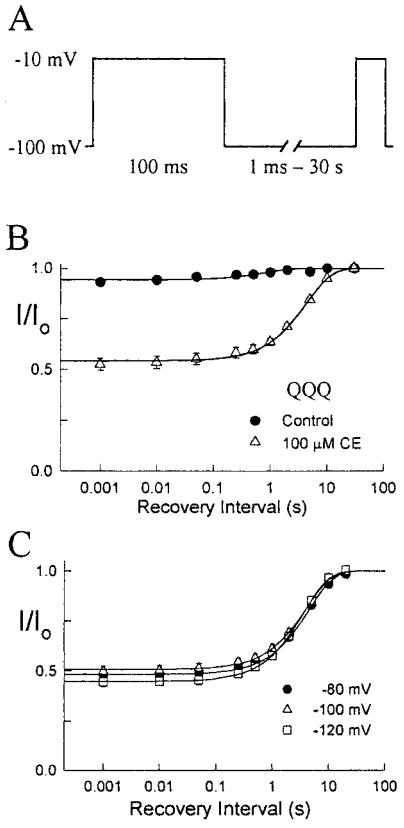

Fig. 7. Recovery of the noninactivating (QQQ) mutant channel. A, pulsing protocol employed to measure the recovery of drug-modified QQQ mutant channels. Test currents were normalized (I/I_o) to current measured after a prolonged rest (60 s) at −100 mV (I_o) and plotted versus the recovery interval (t). B, recovery time course of the drug-free controls (●) and after application of 100 μM (QQQ) cocaethylene (△). The smooth curves are fits to a single exponential [$I/I_o = 1 - A \times \exp(-t/\tau)$], where τ is the recovery time constant and A is the corresponding relative amplitude. The time constants and relative amplitudes are listed in Table 2. C, the same pulsing protocol was applied as in A except that the recovery voltage was varied between −120 and −80 mV. The smooth curves are exponential curve fits with time constants of 4.6 ± 0.1 s (−80 mV), 4.2 ± 0.1 s (−100 mV), and 3.7 ± 0.1 s (−120 mV) (n = 5).

This contrasts with the QQQ-Y1767C mutation, which weakens cocaethylene binding by selectively increasing the dissociation rate constant (Fig. 6D). After application of cocaethylene, the recovery time course of the QQQ-Y1767C mutant was found to be biexponential, with fast and slow time constants of 0.07 s and 3.4 s, respectively (Fig. 8B). The majority of the channels (67%) recover with the slower time constant, which is similar to what is observed for the recovery of the QQQ mutant (τ = 4.6 s). Although the Y1767C mutation significantly weakens cocaethylene binding, the untrapping from this mutant is only slightly more rapid than the QQQ control. This suggests that dissociation from the cytoplasmic binding site is not a critical determinant of cocaethylene untrapping from the closed channel. The origin of

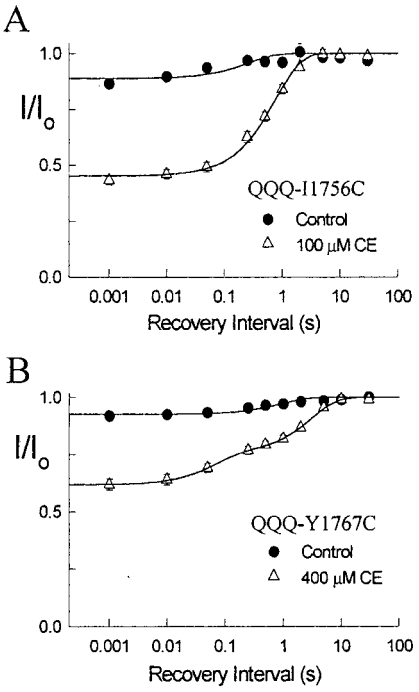

Fig. 8. Untrapping of cocaethylene from the D4S6 mutants. The pulsing protocol was identical to that described in Fig. 7A. A and B, the recovery time course of the drug-free controls (●) and after application of 100 μM (QQQ-I1756C) or 400 μM (QQQ-Y1767C) cocaethylene (△). The smooth curves drawn through the QQQ-I1756C mutant and the QQQ-Y1767C control data are fits to a single exponential (Table 2). After application of cocaethylene, the recovery of the QQQ-Y1767C mutant was better fitted by the sum fast and slow components [$(I/I_o = (1 - 0.62) - (A_F \times \exp(-t/\tau_F) + A_S \times \exp(-t/\tau_S)) + 0.62$], where A is the relative amplitudes and τ the time constants (Table 2). The 0.62 term is the steady-state cocaethylene inhibition induced by the 100-ms depolarizing prepulse.

TABLE 2

Recovery of the non-inactivating (QQQ) mutant channels

The time constants (τ) and the relative amplitudes (A) obtained from curve fitting the recovery time course of the non-inactivating mutant channels (Figs. 7 and 8). The recovery of the QQQ-Y1767C mutant was best fitted by the sum of two exponentials with fast (τ_f) and slow (τ_s) time constants. n indicates the number of experiments.

	τ	A	n
	s		
QQQ	0.7 ± 0.02	0.09 ± 0.01	5
QQQ + CE	4.6 ± 0.3	0.45 ± 0.03	9
QQQ-I1756C	0.1 ± 0.05	0.12 ± 0.02	4
QQQ-I1756C + CE	0.8 ± 0.07	0.52 ± 0.04	11
QQQ-Y1767C	0.9 ± 0.3	0.07 ± 0.01	5
QQQ-Y1767C + CE			
τ_F	0.07 ± 0.01	0.12 ± 0.02	5
τ_S	3.4 ± 0.4	0.24 ± 0.02	

the rapid component of the QQQ-Y1767C mutant recovery is unclear but may reflect the rapid dissociation of cocaethylene as the mutant channels close. One possibility is that the unbinding of cocaethylene may interfere with the closing of the activation gate similar to what has been described previously for tetra-alkylammonium inhibition of potassium channels (Armstrong, 1971; Holmgren et al., 1997). Unfortunately, our whole-cell recordings lack sufficient resolution to investigate the effect of cocaethylene on the deactivation kinetics.

Discussion

In this study, we examined the inhibition of the human cardiac $Na_v1.5$ channel by cocaethylene, a metabolite of cocaine that is synthesized in humans when it is coingested with alcohol. Cocaethylene causes a characteristic use-dependent inhibition but produces little tonic block under resting conditions, consistent with the preferential binding of this drug to the activated and/or inactivated states of the channel. Sustained depolarizations that stabilize the Na^+ channels in inactivated states enhance the cocaethylene inhibition (Fig. 4) and slow the recovery when the voltage is returned to a hyperpolarized potential (Fig. 3). Cocaethylene also causes a hyperpolarizing shift in the steady-state inactivation that is consistent with high-affinity binding to the inactivated state (K_I = 17 μM) and low affinity binding (K_R = 185 μM) to the resting state of the channel (Fig. 1C). The majority of the cocaethylene inhibition is abolished by mutations of the interdomain D3-D4 linker that remove rapid inactivation (Fig. 4C). These findings are consistent with an important role for fast inactivation in cocaethylene binding and are consistent with previous studies of cocaethylene inhibition of the native cardiac Na^+ current (Crumb and Clarkson, 1992).

A rapid component of cocaethylene inhibition persists in the noninactivating (QQQ) mutant channel (Fig. 4C). This component of cocaethylene inhibition primarily develops after the channels have opened, resulting in a slow decay of the current during prolonged depolarization (Fig. 5). The kinetics of this inhibition increase in a concentration-dependent fashion and are consistent with the predictions of a bimolecular interaction with an apparent K_D of 59 μM (Fig. 6C). Cocaethylene seems to rapidly access the cytoplasmic binding site of open channels via the internal aqueous pathway and inhibits the current of the QQQ mutant by a simple pore blocking mechanism (Bennett et al., 1995; Pugsley and Goldin, 1999; Grant et al., 2000; Kimbrough and Gingrich, 2000; O'Leary and Chahine, 2002).

Concentrations of cocaethylene up to 2 μM have been detected in the blood of emergency room patients (Bailey, 1996), and concentrations as high as 5 μM have been reported in post mortem tissues (Jatlow et al., 1991). These concentrations are within the range in which cocaethylene binding to the inactivated state (K_I = 17 μM) is expected to contribute to the inhibition of the $Na_v1.5$ channel. The role of low-affinity cocaethylene binding to open channels (K_D = 59 μM) is less clear. However, cocaine displays a similar pattern of high-affinity binding to inactivated channels (K_D = 3.6 μM) and low-affinity binding to open channels (K_D = 122 μM) (O'Leary and Chahine, 2002). Unlike cocaine binding to inactivated channels, which was determined solely by voltage-dependent channel gating, cocaine binding to the open channel displayed additional voltage sensitivity that we believe arises as a result of electrostatic interaction between the positively charged drug and the membrane electric field (O'Leary and Chahine, 2002). At a depolarized voltage (+40 mV), this additional voltage sensitivity narrowed the gap between the affinities of cocaine binding to the open (K_D = 14 μM) and inactivated states. Although we have not directly measured the voltage sensitivity of cocaethylene binding to the open channel, its chemical structure is similar to that of cocaine, it shares a common binding site on the D4S6 segment, and it has identical mechanisms of action. We predict that the affinity of cocaethylene block will increase at voltages considered to near the peak of the cardiac action potential so that drug binding to both the open and inactivated states contributes to $Na_v1.5$ inhibition. This contrasts with the resting state of the channel, which binds cocaethylene with low affinity (K_R = 185 μM) and therefore probably does not contribute to the cardiotoxic effects of this drug.

Cocaethylene Slows the Recovery of Closed and Inactivated Channels. The slow recovery of drug-modified Na^+ channels at hyperpolarized voltages is a signature effect of many local anesthetics, but the underlying mechanism has not been clearly established. High-affinity binding to inactivated states (K_I = 17 μM) could account for the slow recovery of drug-modified channels at hyperpolarized voltages (Fig. 3B). However, in this study, we also found that the dissociation of cocaethylene from the noninactivating mutant is unusually slow. Channels that were initially blocked by cocaethylene during depolarization only slowly recover at a hyperpolarized voltage (Fig. 7B). This slow recovery cannot be attributed to drug interaction with fast inactivated channels and, because cocaethylene displays low affinity for closed channels, is unlikely to result from an increase in the binding affinity as the voltage is returned to the hyperpolarized potential. Our data suggest that cocaethylene becomes trapped as the channels close, an interpretation that is consistent with the open-channel blocking mechanism. When closed, the activation gate seems to limit cocaethylene access to the cytoplasmic binding site under resting conditions and trap cocaethylene within the vestibule as the channels deactivate. This result is not predicted by models based solely on state-dependent changes in binding affinity, which generally incorporate low-affinity binding to closed channels, a condition that is expected to promote the rapid dissociation of cocaethylene (Hille, 1977; Hondeghem and Katzung, 1977). Other mechanisms, such as the diffusion of cocaethylene out of the internal vestibule when the channels are closed, may also contribute to the slow recovery at hyperpolarized voltages.

I1756C Enhances Cocaethylene Binding and Facilitates Untrapping from Closed Channels. Previous studies have shown that mutations near the extracellular end of the D4S6 segment, a region that includes Ile1756, facilitated the access of externally applied anesthetics to the cytoplasmic binding site (Ragsdale et al., 1994; Qu et al., 1995; Lee et al., 2001; Sunami et al., 2001). We found that the I1756C mutation slightly accelerated the onset of use-dependent inhibition in the wild type (Fig. 2B) and facilitated cocaethylene binding to the noninactivating QQQ mutant (Fig. 6D). Consistent with previous studies, the I1756C mutation seemed to enhance accessibility and promote cocaethylene

binding. In addition, the I1756C mutation caused a 6-fold increase in the apparent rate of cocaethylene untrapping from the QQQ mutant (Fig. 8A). This rapid untrapping cannot be attributed to an increase in the rate of unbinding because the I1756C mutation did not alter the dissociation rate constant and reduced the K_D of cocaethylene binding (Fig. 6D). Rather, the data suggest that the I1756C mutation accelerated the untrapping of cocaethylene from the internal vestibule of the QQQ mutant, which seems to be the rate-limiting step in the recovery when the channel is closed.

By contrast, the I1756C mutation does not alter the time course of recovery from cocaethylene inhibition when expressed in the rapidly inactivating background (Fig. 3C). The I1756C mutation permits cocaethylene to rapidly escape when the channel is closed but seems to have no effect on the recovery when the channel is in the high-affinity inactivated conformation (Fig. 1C). We speculate that despite the more rapid untrapping, the recovery of the I1756C mutant remains slow because the dissociation from the binding site rather than the untrapping predominates when the channel is inactivated.

This conclusion garners additional support from data showing that the Y1767C mutation significantly attenuated the cocaethylene-induced slowing of recovery when expressed in the rapidly inactivating background (Fig. 3D). Unlike the I1756C mutation, Y1767C significantly weakens cocaethylene binding (Fig. 6D). These findings further support the idea that the affinity of cocaethylene binding is an important determinant of the recovery kinetics when the channel is inactivated. This contrasts with the noninactivating mutant, in which the Y1767C produced a comparatively small (2-fold) reduction in the untrapping time constant (Fig. 8B). Untrapping from the closed channel seems to be less sensitive to mutation-induced changes in cocaethylene binding than the recovery of the fast-inactivated channel. One possibility is that when cocaethylene is trapped within the channel, it may not be invariably associated with the cytoplasmic binding site. The conformational changes of the D4S6 segment that promotes cocaethylene binding as the channel activates may reverse as the channel deactivates, causing the drug to dissociate. In the Kcsa bacterial potassium channel, the transmembrane domain 2 helix, which is believed to be the functional equivalent of the S6 segment of the Na^+ channel, seems to rotate and tilt as the channel opens (Liu et al., 2001; Perozo et al., 1999). Similar conformational changes may contribute to the opening and the subsequent enhanced cocaethylene binding of the $Na_v1.5$ channel. Making the assumption that cocaethylene dissociates into the vestibule as the QQQ mutant channel reverts to the low affinity (i.e., closed) conformation would provide an explanation for the paradoxical findings that the I1756C mutation enhances cocaethylene binding but accelerates untrapping. In the wild-type channel, fast inactivation seems to further stabilize cocaethylene binding, hindering its dissociation and thereby preventing rapid untrapping as the channels are returned to a hyperpolarized potential. This would explain why the I1756C mutation has no effect on the recovery of inactivating channels (Fig. 3C). Our data suggest that the slow untrapping from within the internal vestibule of the closed channel and the enhanced binding induced by fast inactivation may represent distinct mechanisms of cocaethylene inhibition.

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