Characteristics of Cocaine Block of Purified Cardiac Sarcoplasmic Reticulum Calcium Release Channels

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ABSTRACT We have examined the effects of cocaine on the SR Ca^{2+} release channel purified from canine cardiac muscle. Cocaine induced a flicker block of the channel from the cytoplasmic side, which resulted in an apparent reduction in the single-channel current amplitude without a marked reduction in the single-channel open probability. This block was evident only at positive holding potentials. Analysis of the block revealed that cocaine binds to a single site with an effective valence of 0.93 and an apparent dissociation constant at 0 mV ($K_d(0)$) of 38 mM. The kinetics of cocaine block were analyzed by amplitude distribution analysis and showed that the voltage and concentration dependence lay exclusively in the blocking reaction, whereas the unblocking reaction was independent of both voltage and concentration. Modification of the channel by ryanodine dramatically attenuated the voltage and concentration dependence of the on rates of cocaine block while diminishing the off rates to a lesser extent. In addition, ryanodine modification changed the effective valence of cocaine block to 0.52 and the $K_d(0)$ to 110 mM, suggesting that modification of the channel results in an alteration in the binding site and its affinity for cocaine. These results suggest that cocaine block of the SR Ca^{2+} release channel is due to the binding at a single site within the channel pore and that modification of the channel by ryanodine leads to profound changes in the kinetics of cocaine block.

INTRODUCTION

The sarcoplasmic reticulum (SR) Ca^{2+} release channel is an important component in the initiation of excitation-contraction coupling in cardiac and skeletal muscle (for a review see Williams, 1992; McPherson and Campbell, 1993; Meissner, 1994). In the heart, calcium influx across the sarcolemma is responsible for the efflux of Ca^{2+} from the SR via the SR Ca^{2+} release channels. This process has been referred to as calcium-induced calcium release (Fabiato, 1983). Studying the behavior of the SR Ca^{2+} release channel has been made possible by incorporating junctional SR membranes into planar lipid bilayers (Smith et al., 1985). Numerous physiological (Ca^{2+}, ATP, Mg^{2+}, calmodulin) and pharmacological (ryanodine, ruthenium red, caffeine) agents that have been demonstrated to affect muscle contractility by influencing the calcium-induced calcium release process have been shown to alter the single-channel activity of the SR Ca^{2+} release channel (for reviews, see Williams, 1992; Meissner, 1994). Purification of the channel from skeletal or cardiac muscle by Chaps solubilization has permitted the examination of monovalent and divalent conduction in the absence of contaminating K^+ or Cl^- channel conductances (Lai et al., 1988; Smith et al., 1988; Liu et al., 1989; Lindsay et al., 1991). The channel displays a broad selectivity for both monovalent and divalent cations but is selectively permeable to divalent cations compared to monovalent species (Smith et al., 1988; Liu et al., 1989; Lindsay et al., 1991). However, the use of monovalent cations as permeant charge carriers has improved the resolution of single-channel events (Smith et al., 1988; Fill et al., 1990; Sitsapesan and Williams, 1994a).

It has been known for many years that the local anesthetic agent procaine inhibits muscle contraction (Feinstein, 1963; Bianchi and Bolton, 1967; Chapman and Miller, 1974) and SR Ca^{2+} release (Feinstein, 1963; Nagasaki and Kasai, 1981; Chamberlain et al., 1984). However, only recently has evidence been provided demonstrating that procaine can directly block the SR Ca^{2+} release channel (Tinker and Williams, 1993a; Xu et al., 1993b; Zahradníková and Palade, 1993). Cocaine, which possesses local anesthetic properties, has also been shown to have a direct negative inotropic action on cardiac muscle (Carpentier et al., 1993; Perreault et al., 1993; Simkhovich et al., 1994). This negative inotropic property of cocaine has been suggested to be the result of inhibition of sarcolemmal ion channels, more notably the TTX-sensitive Na^+ channel and L-type Ca^{2+} channels (Crumb and Clarkson, 1990; Renard et al., 1994). Some investigators have suggested that the primary action of cocaine is to interfere with SR Ca^{2+} release (Carpentier et al., 1993; Tomita et al., 1993), whereas others have suggested otherwise (Stewart et al., 1991; Renard et al., 1994). For that reason, we have decided to examine the effects of cocaine on the single-channel activity of purified canine SR Ca^{2+} release channel.

MATERIALS AND METHODS

Materials

Ryanodine was purchased from Calbiochem (San Diego, CA) and [^{3}H]ryanodine (61.5 Ci/mmol) from DuPont-New England Nuclear (Boston, MA). Phospholipids were purchased from Avanti Polar Lipids (Alabaster,

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AL). Sodium dodecyl sulfate (SDS) and amido black 10B were obtained from BioRad (Hercules, CA). Cocaine hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were obtained from Sigma.

Isolation of junctional SR membrane vesicles

Canine cardiac junctional SR membrane vesicles were isolated according to the methods of Meissner and Henderson (1987). Briefly, mongrel dogs (15–30 kg) were anesthetized with sodium pentobarbital and their hearts were quickly excised. Hearts were placed into an ice-cold 300 mM sucrose solution. The ventricles were trimmed of fat, connective tissue, and major vessels, and cut into smaller pieces. The tissue was homogenized in 7 vol of 300 mM sucrose, 20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (dipotassium salt; K_2 PIPES) (pH 7.4) and 0.5 mM EDTA supplemented with the following protease inhibitors; aprotinin (100 nM), benzamidine (1 mM), iodoacetamide (1 mM), leupeptin (1 μM), pepstatin A (1 μM), and phenylmethylsulfonyl fluoride (PMSF; 1 mM). These protease inhibitors were present in all solutions during the isolation of junctional SR membranes and purification of the SR calcium release channel. The homogenate was centrifuged at 2800 $\times$ g_{max} for 20 min in a Beckman JA-10 rotor. The supernatant was filtered over four layers of cheesecloth and then centrifuged at 120,000 $\times$ g_{max} for 60 min in a Beckman Ti-45 rotor. The sedimented mixed membrane population was resuspended in buffer A: 600 mM KCl, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 10 mM K_2PIPES (pH 7.0), and fractioned on discontinuous sucrose-density gradients. The membrane suspension was layered over 20%, 30%, and 40% (w/v) sucrose in buffer A and sedimented at 120,000 $\times$ g_{max} for 16 h in a Beckman SW28 rotor. Junctional SR membrane vesicles were collected at the 30–40% interface, then diluted in 1.5 vol of 400 mM KCl. The membrane vesicle suspension was pelleted by centrifugation at 120,000 $\times$ g_{max} for 60 min in a Beckman Ti-45 rotor. The pellet was resuspended in a solution containing 300 mM sucrose and 5 mM HEPES titrated to pH 7.2 with tris(hydroxymethyl)aminomethane (Tris). Membrane vesicles were snap frozen and stored in liquid nitrogen. Protein concentrations were determined with a Coomassie protein assay kit (Pierce, Rockford, IL) with bovine serum albumin used as the standard.

Purification and reconstitution of the SR calcium release channel

Purification of the canine cardiac calcium release channel was performed according to the methods of Lai et al. (1988) and Anderson et al. (1989), with modifications described by Lindsay and Williams (1991). Junctional SR membrane vesicles (1–1.5 mg/ml final protein concentration) were solubilized in buffer B: 1 M NaCl, 20 mM Na_2PIPES (pH 7.2), 0.15 mM $CaCl_2$, 0.1 mM EGTA, 5 mM Na_2AMP, 1 mM dithiothreitol, 5 mg/ml phosphatidylcholine, 0.5% (w/v) 3-[3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (Chaps) in the presence of protease inhibitors. [^{3}H]Ryanodine (5 nM) was added to one sample. The samples were incubated for 1 h on ice and then centrifuged at 72,000 $\times$ g_{max} for 30 min in a Beckman Ti-45 rotor. The supernatant was layered on top of six 5–20% (w/w) linear sucrose gradients in buffer B. The gradients were centrifuged at 2°C in a Beckman SW28 rotor at 90,000 $\times$ g_{max} for 16 h. Fractions from the sample containing [^{3}H]ryanodine were collected in 1.25-ml portions from the bottom of the gradient. Aliquots (0.1 ml) were taken and added to 3 ml of scintillation cocktail for determination of the ryanodine receptor peak fraction(s). Identical fractions were taken from the remaining five gradients.

The purified calcium release channels were reconstituted into unilamellar liposomes by dialysis overnight against 1 liter of 0.1 M NaCl, 20 mM Na_2PIPES (pH 7.2), 0.15 mM $CaCl_2$, 0.1 mM EGTA, 1 mM dithiothreitol, and 1 mM PMSF with four volume changes. At the end of dialysis, an equal volume of 0.3 M sucrose, 5 mM HEPES/Tris (pH 7.2) was added to the vesicles. Vesicles were snap frozen and stored in liquid nitrogen.

Protein concentrations were determined by the method of Kaplan and Pederson (1985), using amido black with 0.45 μm Millipore filters (Type HA). Bovine serum albumin was used as the protein standard.

Planar lipid bilayer measurements

Calcium release channel activity was recorded by using the Mueller-Rudin lipid bilayer technique. Lipid bilayers were formed from a suspension of phosphatidylethanolamine and phosphotidylserine (1:1; 40 mg/ml phospholipid in decane) across a 200-μm hole in a Delrin partition that separates the *cis* and *trans* chambers of the bilayer apparatus. Purified calcium release channels were added to the *cis* chamber containing 250 mM KCl, 0.15 mM $CaCl_2$, 0.1 mM EGTA, 10 mM HEPES/Tris (pH 7.4). The *trans* chamber contained a similar solution, except that the concentration of KCl was 50 mM. After channel incorporation, the ionic gradient was equilibrated. The cytoplasmic side of the channel always faced into the *cis* chamber. The *cis* chamber was held at virtual ground and the *trans* chamber could be clamped at various holding potentials relative to ground. This is opposite to the convention used, but this increased the stability of the bilayer when additions to the *cis* chamber were performed. To maintain convention, potentials given are those experienced at the *cis* (cytoplasmic) side relative to the *trans* (luminal) side, such that current flowing at positive holding potentials corresponds to current flowing from the *cis* to *trans* side (Bertl et al., 1992). The Ca^{2+} sensitivity and the orientation of the channel were tested by reducing the free [Ca^{2+}] from 50 μM to 0.1 μM in the *cis* chamber, then increasing it to 30 μM. Free calcium concentrations were calculated using a computer program by Fabiato (1988). Single-channel activity was measured using an Axopatch 200 amplifier and stored directly into a 386 PC using pClamp software (Axon Instruments, Foster City, CA). Data were digitized at 5 kHz and filtered at 2 kHz.

Analysis of cocaine block

The presence of cocaine at the cytoplasmic side of the channel results in flicker block. The single channel alternates between the open and blocked states, but the individual openings are blurred by the limited bandwidth of the patch-clamp amplifier (Hille, 1992). Consequently, the amplitude distribution analysis method described by Yellen (1984) was used to measure the fast blocking and unblocking rates of cocaine block on the channel. This model describes a two-state process (open $\leftrightharpoons$ blocked) with rates α (dissociation rate) and β (association rate), filtered with a first-order filter with a time constant τ. The amplitude distribution of the filtered output (y) is described by the following probability distribution density function $f(y)$ (β distribution):

$$f(y) = y^{a-1}(1 - y)^{b-1}/B(a,b), \tag{1}$$

where

$$B(a,b) = \int_0^1 y^{a-1}(1 - y)^{b-1}\mathrm{d}y \tag{2}$$

and

$$a = \alpha\tau \qquad b = \beta\tau\,. \tag{3}$$

A correction for a 4-pole Bessel filter of -3 dB attenuation frequency, f, is $\tau = 0.350/f$, where τ is the time constant of the equivalent single-pole filter. This model corresponds well with the theory when $a, b > 2$ (see Woodhull, 1973, and Yellen, 1984, for assumptions and limitations concerning the use of this approach).

Amplitude histograms were compiled from single-channel recordings where the channel was open and closed, and normalized to the open-channel current in the absence of cocaine. A gaussian curve was fitted to the baseline (closed) peak and was subtracted from the histogram to remove the closed-channel contribution to the amplitude histogram. The

remaining histogram was fitted with the β distribution by using an iterative, least-squares analysis and was convolved with the gaussian curve. The blocking (k_b) and unblocking (k_{-b}) rate constants were obtained from α and β by using the following equations:

$$k_b = \beta/[B] \quad (4)$$

$$k_{-b} = \alpha. \quad (5)$$

Analysis of cocaine block of ryanodine-modified SR calcium release channels

The addition of cocaine to ryanodine-modified channels results in a slower block of the channel as compared to the unmodified channel, such that open and blocked events could be fully resolved. The half-amplitude threshold analysis (Colquhoun and Sigworth, 1983) was used to determine open and blocked states, and to compile lifetime histograms from single-channel records, which were digitized at 10 kHz and filtered at 2–5 kHz. Lifetime histograms were fitted using pSTAT software (Axon Instruments), which uses Marquardt-Levenberg algorithms for statistical estimates of open and blocked time constants.

RESULTS

Characteristics of purified canine SR Ca^{2+} release channels

Isolation and purification of the SR Ca^{2+} release channel from dog heart resulted in a Ca^{2+}- and ATP-sensitive channel. In the presence of symmetrical 250 mM KCl, elevation of the free Ca^{2+} levels in the *cis* (cytoplasmic) chamber resulted in an increase in single-channel open probability (P_o) (Fig. 1 *A*). At 100 nM free Ca^{2+} (pCa 7), there was an absence of channel activity. Elevation in the free Ca^{2+} concentration to 1 μM resulted in the appearance of very brief openings. A maximum P_o was observed at a free Ca^{2+} level of 30 to 100 μM (P_o ranging from 0.7 to 0.9). This is similar to the Ca^{2+} dependence observed with native canine cardiac SR Ca^{2+} release channels (Rousseau and Meissner, 1989; Chu et al., 1993). Adenine nucleotides have been shown to stimulate Ca^{2+} release from SR membrane vesicles (Meissner, 1984; Meissner and Henderson, 1987) and activate the SR Ca^{2+} release channel (Smith et al., 1985, 1986, 1988). The effect of ATP on the channel when present on the cytoplasmic face of the channel is shown in Fig. 1 *B*. ATP enhanced the single-channel P_o even at very low values of pCa (7 and 6) by increasing the frequency and duration of channel openings, as has been described by others (Smith et al., 1986, 1988).

The neutral plant alkaloid ryanodine has been useful in the study of excitation-contraction coupling in muscle because of its ability to alter SR Ca^{2+} release (Sutko and Kenyon, 1983; Marban and Wier, 1985; Meissner, 1986). Its ability to bind with high affinity to the SR Ca^{2+} release channel (ryanodine receptor) has also facilitated the purification of this channel (Pessah et al., 1986; Imagawa et al., 1987; Inui et al., 1987; Lai et al., 1988). The modification of the gating and conductance of the channel by ryanodine has provided a specific ligand to characterize the SR Ca^{2+} release channel (Rousseau et al., 1987). Fig. 1 *C* illustrates

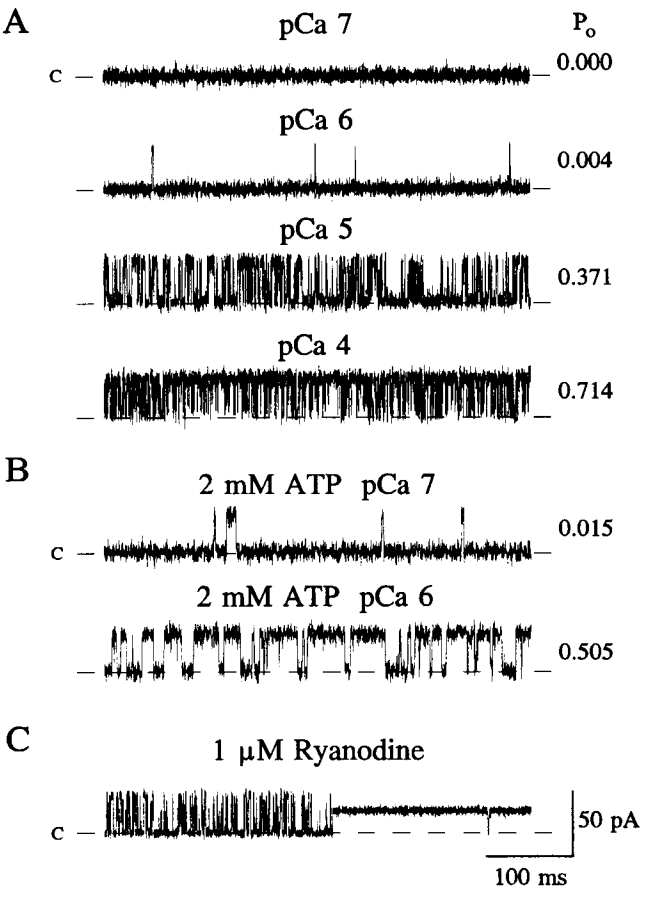

FIGURE 1 Modulation of purified SR Ca^{2+} release channel activity by Ca^{2+}, ATP, and ryanodine. Single-channel activity was recorded under symmetrical 250 mM KCl. (*A*) Enhancement of the single-channel open probability (P_o) by increases in the free Ca^{2+} concentration in the *cis* chamber. (*B*) Increase in single-channel activity in the presence of 2 mM ATP at a free Ca^{2+} concentration of 0.1 or 1 μM in the *cis* chamber. P_o is indicated to the right in *A* and *B*. Records are from a channel separate from that shown in *A*. (*C*) Appearance of the subconductance state in the presence of 1 μM ryanodine. The dashed line represents the zero current level. Holding potential was +40 mV.

the appearance of a long-lived open-state channel with a reduced conductance of ~60% of control after modification by 1 μM ryanodine. In separate experiments, the Ca^{2+} release channel was activated by caffeine (1–5 mM) and was inhibited by 10 μM ruthenium red or 5 mM Mg^{2+} (data not shown). We conclude that the channel we have purified is the SR Ca^{2+} release channel. Our channel displays the same physiological and pharmacological properties as the native channel, suggesting that neither the isolation nor purification processes profoundly modified the channel.

The current-voltage relationship displayed an ohmic slope conductance (γ_K) of 713 ± 4 pS (n = 11) under symmetrical 250 mM KCl (Fig. 2). This is similar to that reported for the cardiac channel purified from dog left ventricle (γ_K = 770 ± 28 pS; Xu et al., 1993a) and septum (γ_K = 713 ± 11 pS; Xu et al., 1993a) and sheep left ventricle (γ_K = 723 ± 9 pS; Lindsay et al., 1991) recorded under similar conditions. The appearance of subconduc-

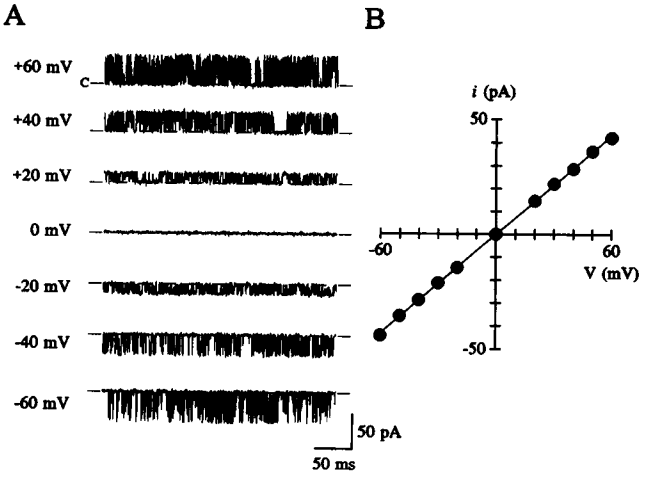

FIGURE 2 (*A*) Single-channel activity at a holding potential range of +60 to −60 mV. Recordings were performed under symmetrical 250 mM KCl solutions (10 μM free [Ca^{2+}]). Holding potentials are as indicated. The dashed line represents the zero current level. (*B*) Single-channel current-voltage relationship for the channel shown in *A*. The linear relationship gives a slope conductance of 715 pS.

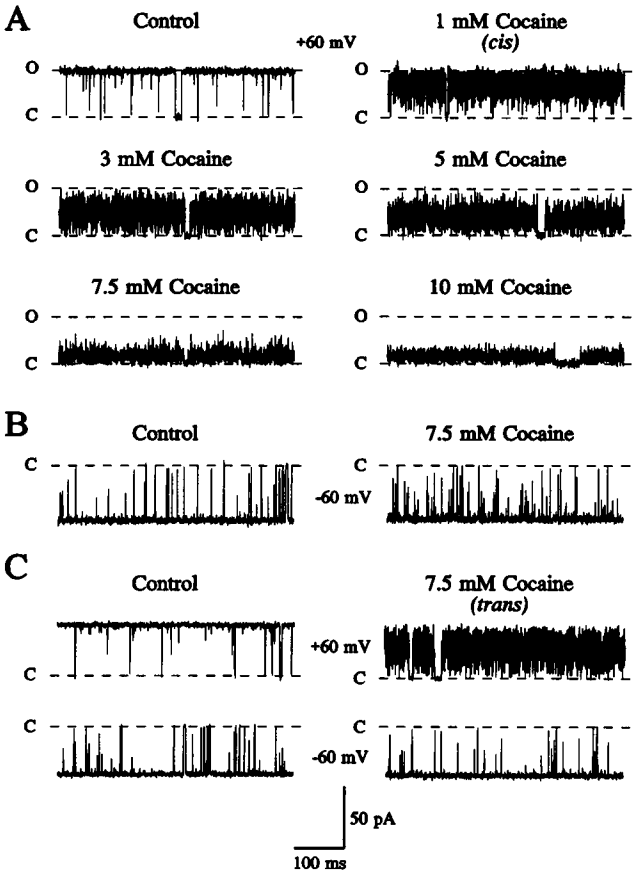

FIGURE 3 Effects of cocaine on single-channel activity of purified SR Ca^{2+} release channels when present in the *cis* or *trans* chamber. (*A*) Enhanced cocaine block of single-channel activity with increasing *cis* concentrations. Control activity was recorded in symmetrical 250 mM KCl solutions (30 μM free [Ca^{2+}]). The dashed lines represent the open (O) and closed (C) current levels. Holding potential was +60 mV. (*B*) Lack of effect of 7.5 mM cocaine at negative holding potentials. Same channel as in *A*. Holding potential was −60 mV. Dashed lines represent the baseline level. (*C*) Cocaine block of single-channel activity when present in the *trans* chamber. Block is only observed at positive holding potentials. Zero current level is represented by the dashed lines.

tance states with the purified SR Ca^{2+} channel has been described by others (Smith et al., 1988; Hymel et al., 1988; Anderson et al., 1989; Liu et al., 1989), although Lindsay and Williams (1991) have reported a reduced incidence of subconductance states when the channel was solubilized in less Chaps detergent (0.5% versus 1.5%) and for a shorter period (1 h versus 2 h). Using their methods, we too observed a very low incidence of subconductance states.

Effects of cocaine on the Ca^{2+} release channel

The effects of local anaesthetic agents on the SR Ca^{2+} release channel have differed, depending on the side of the channel to which each agent is added (Tinker and Williams, 1993a; Xu et al., 1993b). For this reason, we examined the effects of cocaine on single-channel activity when present on either the *cis* or *trans* side of the channel. The effects of cocaine on the *cis* side of the channel are shown in Fig. 3, *A* and *B*. Cocaine elicited a small reduction in the single-channel current amplitude and increased open-channel noise at 1 mM (Fig. 3 *A*). The degree of block was enhanced by increases in the concentration of cocaine in the *cis* chamber. Cocaine induced a flicker block of SR Ca^{2+} release channels. This type of channel block is characterized by brief channel openings that are long enough to detect but too brief to resolve completely (Hille, 1992). Cocaine had no dramatic effect on single-channel P_o. At negative holding potentials, there was an absence of cocaine-induced flicker block (Fig. 3 *B*).

The addition of 7.5 mM cocaine to the *trans* side resulted in a similar block of the SR Ca^{2+} release channel (Fig. 3 *C*); however, the potency of block was much less than that observed when cocaine was added to the *cis* chamber. Under our experimental conditions, 92% of cocaine is in the charged (protonated) form and 8% is in the uncharged form. Thus we considered the possibility that when cocaine is present in the luminal (*trans*) side, channel block may result from the diffusion of uncharged (lipid-soluble) cocaine through the lipid bilayer into the cytoplasmic chamber, where the protonated form of the drug can then block the channel. Although cocaine blocked the channel when present only on the cytoplasmic side, further experiments were performed with cocaine added to both sides of the membrane to avoid asymmetrical surface potentials (Miller, 1982).

Voltage dependence of cocaine block

The degree of cocaine block was enhanced at more positive holding potentials. This is seen in the single-channel cur-

rent-voltage relationships with different cocaine concentrations (Fig. 4). An apparent inward rectification of the current-voltage relationship is observed at positive potentials, with very little effect on the single-channel current amplitude at negative potentials.

Our data suggest that a model for cocaine block of the SR Ca^{2+} release channel involves cocaine binding to a site within the channel pore. A simple scheme of cocaine block can be written as

$$\text{Closed} \leftrightarrows \text{Open} \underset{k_{-b}}{\overset{k_b[\text{cocaine}]}{\rightleftharpoons}} \text{Blocked}.$$

If this model is correct for cocaine block, then the ratio of the single-channel amplitude in the presence of cocaine (i) to that measured in the absence of drug (i_o) is proportional to the time spent in the open state, given by $k_{-b}/(k_{-b} + k_b[\text{cocaine}])$. This can be rearranged to give

$$i/i_o = (1 + ([\text{cocaine}]/K_d))^{-1}, \quad (6)$$

where

$$K_d = k_{-b}/k_b. \quad (7)$$

This equation describes an inverted form of the Langmuir saturation isotherm for ligand binding to a single site. Woodhull (1973) modified this equation and expressed K_d as a Boltzmann relationship

$$K_d = K_d(0)e^{(z\delta FV/RT)}, \quad (8)$$

where $K_d(0)$ is the dissociation rate constant of the blocker at 0 mV, z is the valence of the blocking compound, δ is the fractional electrical distance to the blocking site from the cytoplasmic side, V is the holding potential, and F, R, and T have their usual thermodynamic meanings. Equation 7 can be rearranged to give

$$i/i_o = K_d(0)e^{(z\delta FV/RT)}. \quad (9)$$

From Eq. 9 we can quantitate the blocking behavior of cocaine by plotting the relative single-channel current amplitude (i/i_o) as a function of the holding potential (Fig. 5). The data fit well to Eq. 9 at 5, 7.5, and 10 mM cocaine. The best-fit values for δ and $K_d(0)$ at 5 mM cocaine were 0.93 ± 0.03 and 37.5 ± 3.4 mM (n = 5), respectively. Similar values were obtained at 7.5 mM (0.94 ± 0.01; 38.3 ± 2.8 mM) and 10 mM cocaine (0.93 ± 0.03; 37.5 ± 1.4 mM). These results suggest that cocaine interacts with a site within the channel pore that is equivalent to 93% of the voltage drop from the cytoplasmic side of the channel.

Kinetics for cocaine block

Cocaine elicits a fast flicker block of the channel, which does not allow us to resolve the individual blocking and unblocking events. Using the amplitude distribution analysis (Yellen, 1984) we can derive the blocking (k_b) and unblocking (k_{-b}) rate constants from the amplitude histograms (see Materials and Methods). Fig. 6 shows the single-channel current records at +20, +40, and +60 mV in the presence of 7.5 mM cocaine and the corresponding amplitude histogram fitted to the β distribution. The histograms show the fitted gaussian distribution to the closed current level convolved with the fitted β distribution to the open-channel amplitude. The peak of the fitted β distribution shifts toward the closed current level at more positive potentials. The voltage dependence of the blocking and unblocking rate constants of cocaine is summarized in Fig. 7 for normal (Fig. 7 *A*) and ryanodine-modified channels (Fig. 7 *B*). The voltage dependence of k_b was fitted to a Boltzmann distribution:

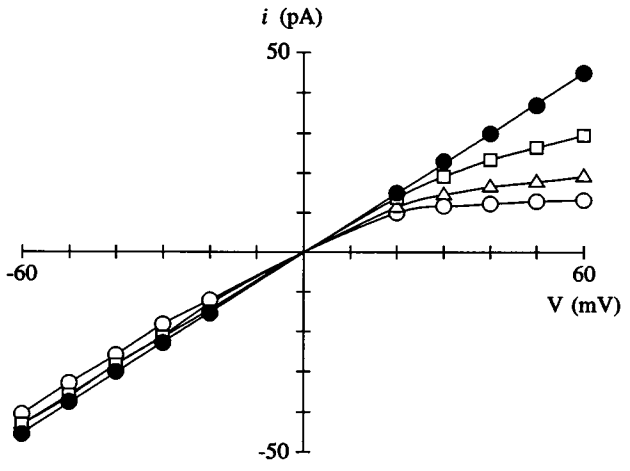

FIGURE 4 Current-voltage relationships of the purified SR Ca^{2+} release channel in the absence (●) or presence of 5 (□), 7.5 (△), or 10 (○) mM cocaine.

$$k_b = k_b(0)e^{(z\delta FV/RT)}, \quad (10)$$

where $k_b(0)$ is the blocking rate constant at 0 mV. The slope corresponds to a fractional electrical distance of 0.92 ± 0.04 (n = 5). The unblocking rate constant displayed no voltage dependence, with a mean $k_{-b}(0)$ of 14.2 ± 0.7 ms^{-1} (n = 5). The dissociation rate constant at 0 mV ($K_d(0)$) derived from Eq. 8 was as 27.8 ± 3.9 mM, similar to that determined by the Woodhull model (38 mM). These results suggest that the voltage dependence of cocaine block lies exclusively in the blocking reaction.

The blocking behavior of cocaine exhibited a concentration dependence, as illustrated in Figs. 3 and 4. The amplitude distribution analysis was also used to determine the blocking (β) and unblocking (α) rates as a function of concentration. The single-channel current activity in the presence of 3, 5, and 7.5 mM cocaine (holding potential = +40 mV) and the corresponding amplitude histogram fitted to the β distribution are shown in Fig. 8. As shown previously, increases in cocaine concentrations reduce the single-channel current ampli-

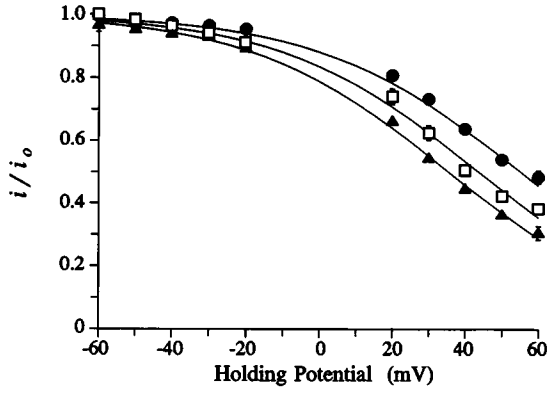

FIGURE 5 Plot of the ratio of the single-channel current amplitude in the presence of cocaine (i) to that measured in the absence of drug (i_o) as a function of the holding potential. Single-channel records were filtered at 200–500 Hz and amplitude histograms were compiled to determine the single-channel amplitude. The cocaine concentrations were 5 (●), 7.5 (□), and 10 (▲) mM. The solid lines represent the best fit to Eq. 9 using an iterative, least-squares analysis method. Parameters for the fit are provided in the text. Each point represents the mean ± SEM of five experiments.

tude, as seen in the single-channel records and by the leftward shift of the peak of the fitted β distribution curve. The concentration dependencies of α and β are shown in Fig. 9 *A*. The blocking rate (β) increased linearly with cocaine concentration with a slope of 3.42 ± 0.22 $mM^{-1}ms^{-1}$, whereas the unblocking rate (α) was unaffected by cocaine concentration (13.7 ± 0.5 ms^{-1}). These data indicate that the enhanced channel block observed with increases in cocaine concentration and voltage are dependent almost exclusively on the blocking rate.

Effect of ryanodine modification on cocaine block

Ryanodine modification of the SR Ca^{2+} release channels results in a channel that is insensitive to cytoplasmic Ca^{2+} and Mg^{2+}, in addition to the characteristic pharmacological changes in gating and conductance (Rousseau et al., 1987). Block of the ryanodine-modified channel by certain local anesthetics also displays a decreased affinity and slower blocking kinetics than the unmodified channel (Tinker and Williams, 1993b; Xu et al., 1993b), which may be due to structural alterations in the conduction pathway of the channel. Fig. 10 *A* illustrates the block by cocaine of a SR Ca^{2+} release channel modified with 1 μM ryanodine. In the presence of 3 mM cocaine (holding potential +40 mV), discrete open and blocked events could be resolved. An enhancement of channel block was observed with increasing concentrations of cocaine. The degree of block was augmented as the holding potential was held more positive (Fig. 10 *B*). As observed in the unmodified channel, cocaine did not block ryanodine-modified channels at negative potentials. The single-channel current amplitude of the ryanodine-modified channel was not altered by cocaine.

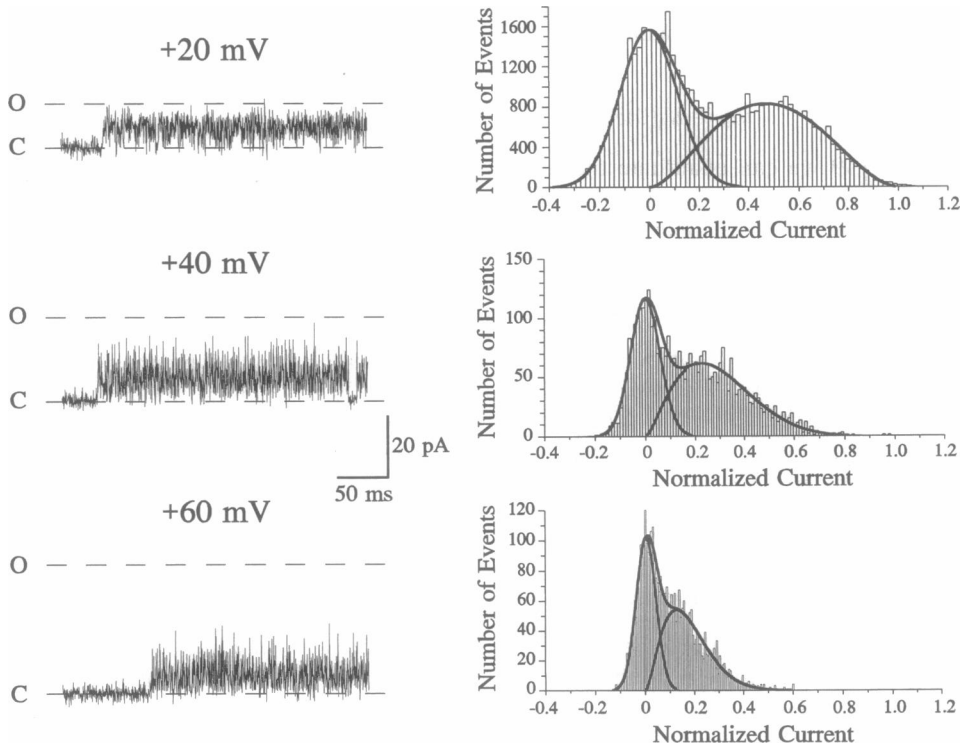

FIGURE 6 Amplitude distribution analysis of the voltage dependence of cocaine block. Representative single-channel records at +20, +40, and +60 mV in the presence of 7.5 mM cocaine are shown on the left. The open and closed current amplitude levels are indicated by the dashed lines. The open current level indicates the single-channel amplitude in the absence of cocaine. The corresponding amplitude distribution analysis is on the right. The amplitude histograms were constructed and analyzed as described in Materials and Methods. The best fits to the β function were a = 2.72 and b = 2.94 at +20 mV; a = 2.51 and b = 6.17 at +40 mV; and a = 2.74 and b = 13.10 at +60 mV.

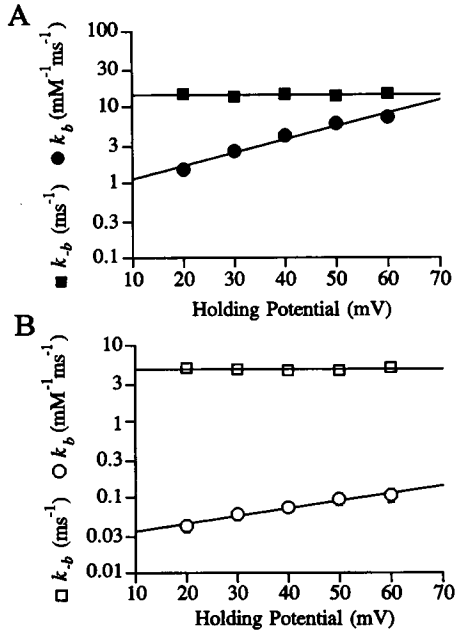

FIGURE 7 The voltage dependence of the blocking (k_b) and unblocking (k_{-b}) rate constants for cocaine block of (*A*) control and (*B*) ryanodine-modified channels. The data were fitted as described in the Results. The symbols represent the mean ± SEM of five experiments for unmodified channels and four experiments for ryanodine-modified channels.

Kinetics of cocaine block of ryanodine-modified channels

The kinetics of cocaine block of ryanodine-modified channels were measured using the open- and blocked-state lifetime histograms. Lifetime histograms from single-channel recordings of ryanodine-modified channels in the presence of 7.5 mM cocaine are presented in Fig. 11. These lifetime histograms were well described by a single exponential function. The open (τ_o) and blocked (τ_b) time constants were used to calculate k_b and k_{-b}, respectively:

$$k_b = 1/(\tau_o[\text{cocaine}]) \quad (11)$$

$$k_{-b} = 1/\tau_b \quad (12)$$

Fig. 7 *B* shows the rate constants for cocaine block and unblock measured over a voltage range of +20 to +60 mV. As with the ryanodine-unmodified channel, k_b increased exponentially with voltage, whereas k_{-b} was unaffected by voltage with a mean value of 4.8 ± 0.1 ms^{-1} (n = 4). Analysis of the voltage dependence of k_b by a Boltzmann distribution revealed a δ of 0.52 ± 0.08. This is different from the ryanodine-unmodified channel, where the calculated δ was 0.92 ± 0.04. As with the unmodified channel, the voltage dependence of cocaine block of the ryanodine-modified channel lies in the blocking reaction; however, the blocking site occurs only 52% into the electrical field of the membrane.

The concentration dependence of the blocking and unblocking rate constants in ryanodine-modified channels is shown in Fig. 9 *B*. The dissociation rate (α) of cocaine from the ryanodine-modified channel was not influenced by concentration, as observed with the unmodified channel, although it did decrease to 5.06 ± 0.27 ms^{-1} from 13.7 ± 0.5 ms^{-1}. A more dramatic decrease was observed in the concentration dependence of β. The association rate increased

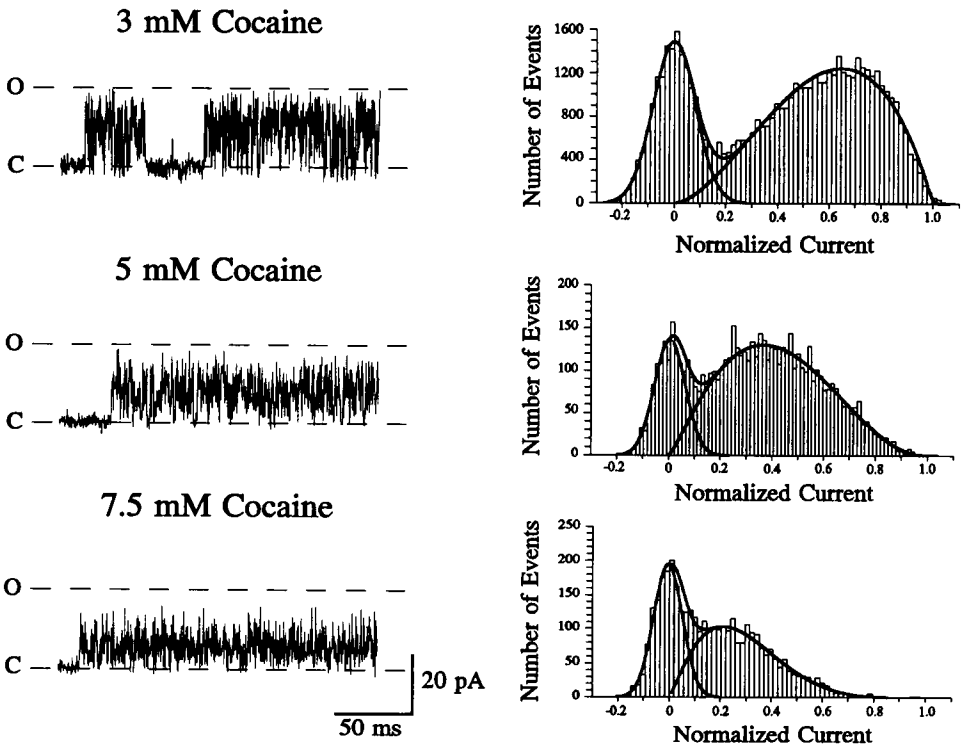

FIGURE 8 Amplitude distribution analysis of the concentration dependence of cocaine block. Representative single-channel activity in the presence of 3, 5, and 7.5 mM cocaine are shown on the left. The holding potential was +40 mV. The open current level indicates the single-channel amplitude in the absence of cocaine. The right panel shows the corresponding amplitude distribution analysis. The amplitude histograms were compiled and analyzed as described in the Materials and Methods. The best fits to the β function for the following concentrations of cocaine were (3 mM) a = 2.75 and b = 2.05, (5 mM) a = 2.28 and b = 3.20, and (7.5 mM) a = 2.24 and b = 5.63.

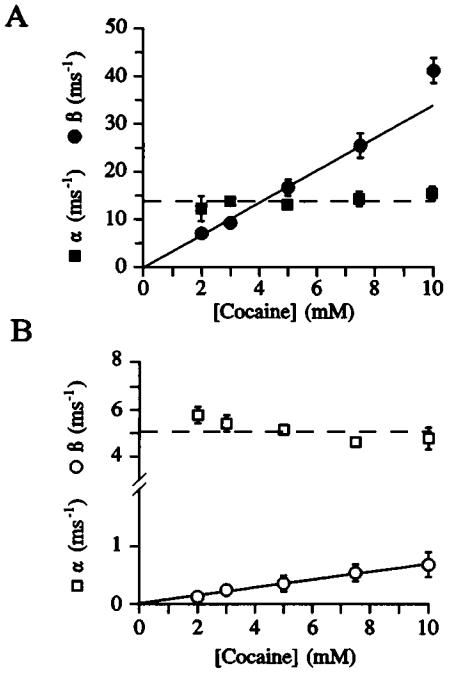

FIGURE 9 The concentration dependence of the blocking (β) and unblocking (α) rates for cocaine block of (*A*) control and (*B*) modified channels. The data were fitted as described in the Results. The symbols represent the mean ± SEM of five experiments for control channels and four experiments for ryanodine-modified channels.

linearly with the cocaine concentration with a slope of 0.072 ± 0.01 $mM^{-1}ms^{-1}$, which was far less than a slope of 3.42 ± 0.22 $mM^{-1}ms^{-1}$ measured in the unmodified channel. Furthermore, the affinity of cocaine for the channel after modification by ryanodine was reduced threefold to $K_d(0)$ of 110.0 ± 7.5 mM from 27.8 ± 7.5 mM in the absence of ryanodine. These results demonstrate that the concentration dependence of the cocaine block of ryanodine-modified channels is dependent on the on rate of cocaine binding and is independent of the off rate as observed in the unmodified channels. In comparison to the unmodified channel, ryanodine modification causes both a modest reduction in the unblocking rate of cocaine and an extensive reduction in the blocking rate.

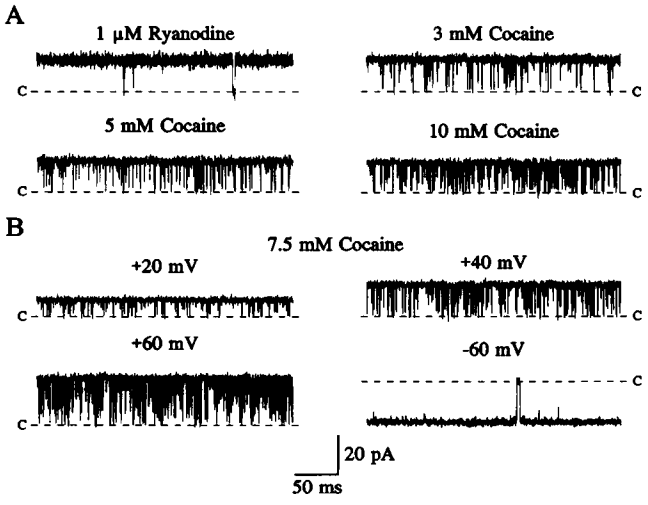

FIGURE 10 Concentration and voltage dependence of cocaine on single-channel activity of ryanodine-modified SR Ca^{2+} release channels. (*A*) Single-channel records of ryanodine-modified channels in the absence and presence of 3, 5, and 10 mM cocaine. The holding potential was +40 mV. (*B*) Enhanced cocaine block with increasing positive holding potentials. Cocaine concentration was 7.5 mM. At −60 mV, no block was observed. The zero or closed current (C) level is indicated by the dashed line in each panel.

DISCUSSION

We have purified the SR Ca^{2+} release channel from canine myocardium, which displayed characteristics typical for the cardiac SR Ca^{2+} release channel, including a high degree of Ca^{2+} sensitivity as well as modulation by ATP, Mg^{2+}, and ruthenium red. In addition, a unique and important characteristic of this channel is modification by ryanodine, which induced a subconductance state of about 60% of normal conductance in our channel after exposure to 1 μM ryanodine. The purified form of our channel also exhibits the same properties as demonstrated with the native channel (Rousseau et al., 1987; Rousseau and Meissner, 1989; Chu et al., 1993). Therefore we believe that the channel we have isolated and purified is similar to that identified by others as the SR Ca^{2+} release channel/ryanodine receptor and has not been dramatically altered during the purification process.

Voltage dependence of cocaine block

Cocaine caused a flicker block of the SR Ca^{2+} release channel when present on the *cis* (cytoplasmic) face of the channel at positive potentials where current flow is from the cytoplasmic to the luminal side. Cocaine did produce a weaker block of the channel when added to the *trans* side of the channel, but again only when current was flowing from *cis* to *trans*. Under our recording conditions, approximately 92% of the drug is in the protonated form, with the remainder in the uncharged form. It is most probable that the uncharged form of cocaine is capable of diffusing through the lipid membrane and gains access to the channel pore from the cytoplasmic side, where it can then initiate channel

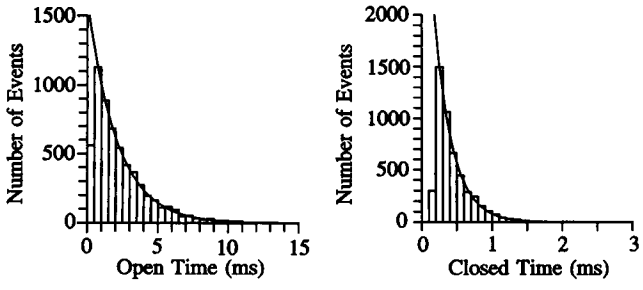

FIGURE 11 Lifetime distributions of the open and closed times of ryanodine-modified channels in the presence of 7.5 mM cocaine at a holding potential of +60 mV. The data were best fit to a single exponential function with an open τ of 2.071 ms and a blocked τ of 0.255 ms.

block. This is further supported by the findings that the quaternary lidocaine derivatives, QX222 and QX314, did not affect SR Ca^{2+} release channel gating or kinetics when present in the *trans* chamber (Tinker and Williams, 1993a; Xu et al., 1993b).

The flicker block produced by cocaine was voltage and concentration dependent. Only when the holding potential was held at positive potentials did cocaine induce block. The block could be well described by the two-barrier, one-site model (Woodhull, 1973) in which cocaine interacted at a fractional electrical distance of 0.93 from the cytoplasmic side. A simple interpretation of this result is that cocaine impedes ion conduction by penetrating the channel pore to a site at 93% of the electric field from the cytoplasmic side. The voltage dependence of cocaine block resides exclusively in the blocking rate, whereas the unblocking rate was unaffected by voltage. This asymmetrical voltage dependence has been interpreted to be the result of a difference in the charge of the species involved in the blocking and unblocking reactions (Moczydlowski, 1986). The complex that forms between the positively charged blocker and the negatively charged binding site in the channel pore may reduce the charge, resulting in a voltage-independent dissociation rate. A second explanation for the lack of voltage dependence of the unblocking rate may result from a diffusional limitation process (Yellen, 1984). This model suggests that there is a decrease in cationic occupancy (in this case K^+) with a concomitant increase in cocaine concentration at the channel mouth at positive potentials. This process would only affect the entry rate of cocaine and not the exit rate. However, this model also predicts that the current-voltage relationship under control conditions should demonstrate sublinearity at extreme potentials because of the depletion of the charge carrier around the mouth of the channel. Our observations only show a linear current-voltage relationship from -60 to $+60$ mV. Although we were limited in the voltage range we were capable of studying due to the stability of the lipid bilayer, we have occasionally examined the current-voltage relationship between -100 to $+100$ mV and observed no sublinearity. This would suggest that the diffusional limitation model may not apply under our experimental conditions.

Recently, Tinker and Williams (1993a) have demonstrated similar findings on the effects of the local anesthetic agents procaine and QX222 on purified sheep SR Ca^{2+} channels. Both agents blocked the channel with an effective valence of 0.9, in a voltage- and concentration-dependent manner similar to that of cocaine. In contrast to the result with procaine, cocaine did not elicit a marked reduction in single-channel P_o, as observed by other investigators (Tinker and Williams, 1993a; Xu et al., 1993b; Zahradníková and Palade, 1993). This effect of procaine has been suggested to be the result of drug interaction with a site on the channel located in or near the lipid membrane (Xu et al., 1993b) and possibly with the long-lived closed state of the channel (Zahradníková and Palade, 1993).

In skeletal Na^+ channels incorporated into planar lipid bilayers' procaine elicits two modes of channel block: a fast-blocking action and a discrete long-lived blocked state (Moczydlowski et al., 1986). Fast block arises from an interaction of procaine with the cytoplasmic region of the pore. The slow-blocking mode can be initiated when procaine is present on either the internal or external side of the channel, suggesting a binding site located within the hydrophobic region of the channel. It was proposed that the two blocking modes were mediated as a result of drug binding to two different sites. Cocaine has been shown only to elicit a fast block of the Na^+ channel (Wang, 1988, 1990). These findings are quite similar to the observations with local anesthetic agents on the SR Ca^{2+} release channel, implying the possibility of two binding sites for local anesthetic agents on the channel. Although our data suggest that cocaine is capable of diffusing across the lipid membrane, the lack of any effect on single-channel P_o may be due to a low affinity of cocaine for the slow blocking site.

Cocaine interactions with the ryanodine-modified channel

Modification of the SR Ca^{2+} release channel by ryanodine has been shown to alter its sensitivity to Ca^{2+}, Mg^{2+}, ATP, and ruthenium red (Rousseau et al., 1987). Indeed, we found that ryanodine modification of the channel resulted in a profound reduction in the voltage and concentration dependence of cocaine block. The on rate of cocaine binding was dramatically reduced from 14.6 $mM^{-1}ms^{-1}$ in the absence of ryanodine to 0.11 $mM^{-1}ms^{-1}$ in the presence of ryanodine (holding potential +60 mV, 7.5 mM cocaine). The off rate also decreased in the presence of ryanodine but to a lesser extent (13.7 ms^{-1} vs 5.1 ms^{-1}). Furthermore, the modification of the channel by ryanodine was also evident in the attenuation of the concentration dependence of the on rate. The linear increase in the on rate with cocaine had a slope of 3.42 $mM^{-1}ms^{-1}$, which was reduced to 0.072 $mM^{-1}ms^{-1}$ after ryanodine modification. The off rate was less markedly affected. Ryanodine-modified SR Ca^{2+} release channels have been shown to reduce the on rate, and to a smaller extent, the off rate of QX314 (Xu et al., 1993b) and tetrabutylammonium (TBA) (Tinker and Williams, 1993b) binding.

Ryanodine modification of the channel also resulted in a decrease in the effective valence of cocaine binding from 0.93 to 0.52. Ryanodine may narrow the vestibule of the channel pore and prevent cocaine from binding to the site deep in the membrane field but still allow the agent to bind to other lower affinity sites within the channel pore. This latter site may be related to the site involved in tetramethylammonium block of sheep SR Ca^{2+} release channels ($\delta = 0.54$) (Tinker et al., 1992a). Streaming potential stud-

ies of the SR Ca^{2+} release channel have demonstrated an increased permeability to Tris and reduced divalent selectivity after ryanodine modification (Tu et al., 1994). These changes were suggested to result from a widening of the channel pore by ryanodine. A similar conclusion was reached by Lindsay et al. (1994) to explain alterations in ion conductance after ryanodine modification. Using a single-ion occupancy Eyring rate theory model, these investigators concluded that ryanodine modification results in conformational alterations in the structure of the channel, leading to changes in the energy profiles sensed by the conducting cations. Such alterations included a widening of the selectivity filter, an increased affinity of the cation binding site located 50% into the voltage drop from the cytoplasmic side, a decreased density of the negative charges lining the pore, and a decrease in the capture radius of the channel. Although no information was presented on the cation binding site located 90% into the voltage drop, these profound changes may explain the alteration in the apparent binding site for cocaine after ryanodine modification of the channel.

Alterations in the effective valence of cocaine binding after channel modification by ryanodine may be the result of screening of the cocaine binding site by ryanodine. Localization of the [^{3}H]ryanodine binding site on the skeletal muscle Ca^{2+} release channel was shown to be in the carboxyl-terminal portion of the channel (Callaway et al., 1994). This region has been proposed to form the ion conduction pathway of the channel according to molecular cloning studies (Takeshima et al., 1989; Otsu et al., 1990; Zorzato et al., 1990). The similarity of the Ca^{2+} dependence of ryanodine binding and channel activation (Pessah et al., 1985; Michalak et al., 1988; Rousseau and Meissner, 1989) suggests that ryanodine is capable of binding only to the open state of the SR Ca^{2+} release channel and possibly within the channel pore. It is possible that ryanodine interferes with cocaine binding by screening the binding site deep in the conduction pathway, thus preventing cocaine from interacting with this site.

The change in the effective valency of cocaine binding in the presence of ryanodine differs from the observations by Tinker and Williams (1993b) with TBA. These investigators observed no alteration in the location of TBA binding in the electric field after ryanodine modification. Interestingly, the effective valency of QX314 binding increased after ryanodine modification of skeletal SR Ca^{2+} release channels (Xu et al., 1993b). It is not known why the observations of the effective valencies differ between the present and above-mentioned studies. However, the modes of drug binding for cocaine, TBA, and QX314 differ. Whereas our observations suggest that a single cocaine molecule binds to a site within the channel pore, TBA induces a more complex block by producing a subconductance state of the channel, the characteristics of which suggest that up to four TBA molecules may be involved in channel block (Tinker et al., 1992b). Two QX314 molecules have been suggested to be involved in block of skeletal SR Ca^{2+} release channels (Xu et al., 1993b). These differences in drug block may in turn be affected differently by ryanodine modification.

An alteration in the binding site for local anesthetic block was observed in batrachotoxin-modified Na^+ channels compared to unmodified channels. Batrachotoxin (BTX) increases the single-channel openings and reduces single-channel conductance by altering Na^+ channel inactivation and ionic selectivity (Huang et al., 1982, 1984; Shenkel et al., 1989). These effects are most likely the result of alterations in the structure of the channel protein (Garber and Miller, 1987; Garber, 1988). Internal block of BTX-activated Na^+ channels by QX-314 revealed a binding site at 36–48% of the electric field from the cytoplasmic side (Moczydlowski et al., 1986; Wang, 1988). Analysis of QX-314 block of BTX-unmodified Na^+ channels, where fast inactivation was removed by papain, demonstrated QX-314 interacted with a deeper site located 70% across the voltage drop from the cytoplasmic opening of the channel (Gingrich et al., 1993). Alterations in the structural configuration of the Na^+ channel pore appear to influence the blocking behavior of local anesthetics. Changes in the SR Ca^{2+} release channel protein induced by ryanodine may explain why local anesthetic agents interact differently with the ryanodine-unmodified versus the modified channel.

In conclusion, we have demonstrated that cocaine induces a flicker block of purified cardiac SR Ca^{2+} release channels in a voltage- and concentration-dependent manner. Cocaine appears to interact with a single site within the channel pore, 93% across the electrical field. Modification of the channel by ryanodine resulted in a slower block with a diminished dependence on voltage and cocaine concentration, which may be manifested by the alteration in the blocking site for cocaine (50% across the voltage drop sensed by the channel) and a reduction in the affinity of the channel binding site for cocaine. This suggests that ryanodine-modification alters cocaine from binding in the channel pore.

Although cocaine is capable of eliciting block on the SR Ca^{2+} release channel, the lower affinity of cocaine for the channel (millimolar) compared to the levels reported in overdose victims (micromolar) (Van Dyke et al., 1976; Isner et al., 1986) or those used to demonstrate a direct negative inotropic effect on cardiac tissues (Stewart et al., 1991; Carpentier et al., 1993; Tomita et al., 1993; Renard et al., 1994; Simkhovich et al., 1994) makes it unlikely that the SR Ca^{2+} release channel is a prominent site of action for this agent. It is possible that we may observe different blocking parameters if we use the more physiological permeant cation, calcium, because it appears to influence how some agents modulate this channel (Sitsapesan and Williams, 1994b, 1995). Further studies are required to examine the influence of the permeating species on SR Ca^{2+} release channel block. Regardless of the biological significance of cocaine block, our present results provide a useful probe for studying the SR Ca^{2+} release channel.

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