Effects of Local Anesthetics on Single Channel Behavior of Skeletal Muscle Calcium Release Channel

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ABSTRACT The effects of the two local anesthetics tetracaine and procaine and a quaternary amine derivative of lidocaine, QX314, on sarcoplasmic reticulum (SR) Ca^{2+} release have been examined by incorporating the purified rabbit skeletal muscle Ca^{2+} release channel complex into planar lipid bilayers. Recordings of potassium ion currents through single channels showed that Ca^{2+}- and ATP-gated channel activity was reduced by the addition of the tertiary amines tetracaine and procaine to the *cis* (cytoplasmic side of SR membrane) or *trans* (SR lumenal) side of the bilayer. Channel open probability was lowered twofold at tetracaine and procaine concentrations of ~150 μM and 4 mM, respectively. Hill coefficients of 2.0 and greater indicated that the two drugs inhibited channel activity by binding to two or more cooperatively interacting sites. Unitary conductance of the K^+-conducting channel was not changed by 1 mM tetracaine in the *cis* and *trans* chambers. In contrast, *cis* millimolar concentrations of the quaternary amine QX314 induced a fast blocking effect at positive holding potentials without an apparent change in channel open probability. A voltage-dependent block was observed at high concentrations (millimolar) of tetracaine, procaine, and QX314 in the presence of 2 μM ryanodine which induced the formation of a long open subconductance. Vesicle–$^{45}Ca^{2+}$ ion flux measurements also indicated an inhibition of the SR Ca^{2+} release channel by tetracaine and procaine. These results indicate that local anesthetics bind to two or more cooperatively interacting high-affinity regulatory sites of the Ca^{2+} release channel in or close to the SR membrane. Voltage-dependent blockade of the channel by QX314 in the absence of ryanodine, and by QX314, procaine and tetracaine in the presence of ryanodine, indicated one low-affinity site within the conduction pathway of the channel. Our results further suggest that tetracaine and procaine may primarily inhibit excitation–contraction coupling in skeletal muscle by binding to the high-affinity, regulatory sites of the SR Ca^{2+} release channel.

INTRODUCTION

In skeletal muscle, the release of calcium ions from the intracellular membrane compartment, sarcoplasmic reticulum (SR), is triggered by an action potential that

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originates at the neuromuscular synapse and spreads rapidly over the muscle surface and into the cell interior through invaginations, the transverse tubule (T-tubule) (Ebashi, 1976; Stephenson, 1981). The electrical excitatory signal is thought to be communicated from the T-tubule to the SR at junctional locations where large protein structures span the narrow gap separating the two membranes (Peachey and Franzini-Armstrong, 1983). These structures have been variously termed "feet" (Franzini-Armstrong, 1970), "bridges" (Somlyo, 1979), "pillars" (Eisenberg and Eisenberg, 1982), or "spanning proteins" (Caswell and Brandt, 1989), and are now believed to be identical with the ligand-gated, ryanodine-sensitive "Ca^{2+} release" channel of SR (for reviews see Fleischer and Inui, 1989; Lai and Meissner, 1989; but see also Zaidi, Lagenaur, Hilker, Xiong, Abramson, and Salama, 1989). The specific mechanism of signal transduction from the T-tubule to SR, commonly referred to as excitation–contraction (E-C) coupling, remains to be fully defined, however. One postulated mechanism is that T-tubule depolarization increases the concentration of a chemical messenger such as Ca^{2+} (Endo, 1977; Fabiato, 1983) or IP_3 (Donaldson, Goldberg, Walseth, and Huetteman, 1988; but see also Walker, Somlyo, Goldman, Somlyo, and Trentham, 1987) in the junctional gap, which then opens the Ca^{2+} release channel in the SR membrane. Another mechanism, the mechanical coupling mechanism, suggests that small transient charge movements observed during T-tubule depolarization may represent the physiological link between T-tubule depolarization and SR Ca^{2+} release (Schneider, 1981). An important implication of the mechanical coupling model is that the SR Ca^{2+} release channel is in direct contact with a voltage-sensing molecule in the T-tubule membrane. Recent biophysical, pharmacological, and molecular biological evidence has suggested that the dihydropyridine (DHP) receptor may be the voltage sensor in E-C coupling in skeletal muscle (Rios and Pizarro, 1988; Tanabe, Beam, Powell, and Numa, 1988).

The two local anesthetics procaine and tetracaine have been used to study the mechanism of E-C coupling in amphibian (Lüttgau and Oetliker, 1968; Almers and Best, 1976; Hui, 1983; Vergara and Caputo, 1983; Csernoch, Huang, Szucs, and Kovacs, 1988) and mammalian (Lamb, 1986) skeletal muscle. Studies with intact or cut (Almers and Best, 1976; Hui, 1983; Vergara and Caputo, 1983), and skinned (Ford and Podolsky, 1972; Thorens and Endo, 1975; Donaldson et al., 1988; Pike, Abramson, and Salama, 1989) fibers as well as isolated membrane preparations (Nagasaki and Kasai, 1981; Kirino and Shimizu, 1982; Morii and Tonomura, 1983; Antoniu, Kim, Morii, and Ikemoto, 1985; Ohnishi, 1987; Palade, 1987; Pike et al., 1989) have indicated that tetracaine and procaine inhibit the release of Ca^{2+} from SR. In most studies a relatively high procaine concentration of 10 mM was used to inhibit SR Ca^{2+} release. In contrast, tetracaine concentrations as low as 10–50 μM were found in some studies to affect SR Ca^{2+} release (Csernoch et al., 1988; Nasri-Sebdani, Traore, Cognard, Potreau, Poindessault, and Raymond, 1990), although in general, concentrations > 100 μM were required to more fully inhibit the SR Ca^{2+} release pathway. Interpretation of the studies using elevated concentrations of tetracaine is complicated because tetracaine, in addition to inhibiting SR Ca^{2+} release, appears to influence T-tubule charge movement (Huang, 1982; Hui, 1983; Csernoch et al., 1988; Pizarro, Csernoch, Uribe, Rodriguez, and Rios, 1991; Szucs, Csernoch, Magyar, and Kovacs, 1991). Additional local anesthetics reported to

inhibit $^{45}Ca^{2+}$ release from isolated SR include the neutral local anesthetic benzocaine, other tertiary amines such as lidocaine or dibucaine, and QX314, a water-soluble quaternary amine derivative of lidocaine (Volpe, Palade, Costello, Mitchell, and Fleischer, 1983; Fernandez-Belda, Soler, and Gomez-Fernandez, 1989).

In this report we have more directly studied the interaction of the SR Ca^{2+} release channel with the two local anesthetics tetracaine and procaine, as well as QX314, by reconstituting the purified 30S rabbit skeletal muscle channel complex into planar lipid bilayers. The reconstituted Ca^{2+} release channel conducted monovalent cations and Ca^{2+} ions, was activated by Ca^{2+} and ATP, and was modified by ryanodine as previously reported (Smith, Coronado, and Meissner, 1986; Lai, Erickson, Rousseau, Liu, and Meissner, 1988; Smith, Imagawa, Ma, Fill, Campbell, and Coronado, 1988). Tetracaine and procaine primarily inhibited the Ca^{2+}- and ATP-gated channel by reducing channel open probability (P_o). In contrast, QX314 reduced single channel conductance without a noticeable effect on channel P_o. An inhibition of the SR Ca^{2+} release channel by micromolar tetracaine and millimolar procaine, but not by millimolar QX314, was also observed using SR vesicle fractions passively loaded with $^{45}Ca^{2+}$.

Some of these results have been communicated in part in abstract form (Xu, Jones, and Meissner, 1990).

EXPERIMENTAL PROCEDURES

Materials

Tetracaine and procaine were obtained from Sigma Chemical Co. (St. Louis, MO), ryanodine was from AgriSystems International (Wind Gap, PA), $^{45}Ca^{2+}$ was from ICN Pharmaceuticals (Irvine, CA), and phospholipids were from Avanti Polar Lipids, Inc. (Birmingham, AL). QX314 was the generous gift of Dr. Bertil Takman (Astra Pharmaceutical Products, Westborough, MA). All other reagents were of analytical grade.

Isolation of SR Vesicles and the 30S Ca^{2+} Release Channel Complex

"Heavy" rabbit skeletal muscle SR vesicles were prepared in the presence of the protease inhibitors diisopropylfluorophosphate (DIFP; 1 mM) and leupeptin (1 μM) as described previously (Meissner, 1984). Briefly, SR vesicles containing the SR Ca^{2+} release channel were recovered from the 36–42% region of sucrose gradients that contained membranes sedimenting at 2,600–35,000 *g*. The 30S Ca^{2+} release channel complex was purified by sucrose density gradient centrifugation after solubilization of heavy SR vesicles in the presence of the zwitterionic detergent CHAPS as previously described (Lai et al., 1988). SDS gel electrophoresis showed the presence of a single high molecular weight protein in Ca^{2+} release channel peak fractions. This suggested that minimal proteolysis of Ca^{2+} release channel polypeptides had occurred during purification.

$^{45}Ca^{2+}$ Flux Measurements with Passively Loaded Vesicles

$^{45}Ca^{2+}$ efflux rates from vesicles passively loaded with $^{45}Ca^{2+}$ were determined by filtration (Meissner, 1984). Unless otherwise indicated, $^{45}Ca^{2+}$ was equilibrated across vesicle membranes (10 mg of protein/ml) by incubation for 2 h at 23°C in a medium containing 20 mM KPipes, pH 7, 100 mM KCl, 0.1 mM EGTA, 1.1 mM $^{45}Ca^{2+}$, 5 μM leupeptin, and 1 mM DIFP. $^{45}Ca^{2+}$ efflux was initiated by diluting vesicles 200-fold into iso-osmolal, unlabeled release media

containing varying concentrations of free Ca^{2+}, AMP, ATP, Mg^{2+}, and local anesthetics. $^{45}Ca^{2+}$ efflux was terminated by the addition of a quench solution (10 mM Mg^{2+}, 1 mM EGTA, and 10 μM ruthenium red, final concentrations) and washing of vesicles with the quench solution on 0.45 μm HAWP filters (Millipore Corp., Bedford, MA) to separate away untrapped and released $^{45}Ca^{2+}$. Radioactivity retained by the vesicles on the filters was determined by liquid scintillation counting. $^{45}Ca^{2+}$ efflux measurements were carried out at least in duplicate using two or more preparations. Standard deviations were ±15% or less.

Planar Lipid Bilayer Measurements

Single channel measurements were performed by incorporation of the purified rabbit skeletal muscle 30S Ca^{2+} release channel complex into Mueller-Rudin planar bilayers containing phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine in the ratio 5:3:2 (50 mg/ml phospholipid in *n*-decane; Lai et al., 1988). Unless otherwise indicated, a symmetrical solution of buffered KCl (0.25 M KCl, 20 mM KPipes, pH 7) was used to record single channel currents. Small aliquots (1–3 μl) of the 30S gradient fractions were added to the *cis* chamber (defined as the SR cytoplasmic side; Miller, 1978) of the planar lipid bilayer apparatus. Both chambers were briefly stirred (10–15 s) after the addition of local anesthetics. In perfusion experiments, local anesthetics were removed by perfusing the chamber containing the local anesthetic with 3–4 vol of a drug-free solution for ~7 min with the use of a Harvard perfusion pump. The purified ryanodine receptor exhibits multiple conductance states when reconstituted into the planar bilayer (Lai et al., 1988; Ma, Fill, Knudson, Campbell, and Coronado, 1988; Smith et al., 1988; Liu, Lai, Rousseau, Jones, and Meissner, 1989). In this study we examined only the fully conducting channel state (770 pS with 250 mM K^+) since this state was seen in a majority (~80%) of our single channel recordings. The electrical signals were filtered at 4 kHz for storage on videotape using a modified audio processor and videotape recorder (Bezanilla, 1985). Unless otherwise indicated, current recordings were filtered at 600 Hz through an eight-pole, low-pass Bessel filter (model 902-LPF; Frequency Devices Inc., Haverhill, MA) and digitized at 2.0 kHz for analysis using a Dell 200 AT-clone computer and a modified version of a program kindly provided by Dr. H. Affolter. P_o values were calculated from the digitized data using a single threshold level set at 50% of the current amplitude (Smith et al., 1986). The P_o values and "diary" plots were determined from data stored in two and five to six 17-s files, respectively. All recordings in this study lasted at least half an hour.

Biochemical Assays

Protein was determined by the method of Kaplan and Pederson (1985) using Amido Black and 0.45-μM filters (type HA; Millipore Corp.). Bovine serum albumin was used as the protein calibration standard. Free Ca^{2+} concentrations were calculated using a computer program and the binding constants published by Fabiato (1981). H^+ concentration measurements with a pH electrode in media containing 0.1 and 0.25 M KCl indicated for tetracaine a pKa of 8.5 and 8.6, respectively, in good agreement with a reported pKa of 8.5 (Courtney, 1980).

RESULTS

Vesicle $^{45}Ca^{2+}$ Flux Measurements

The three compounds used in this study differ in several respects in their physicochemical properties. Tetracaine and procaine are tertiary amines that are largely protonated at pH 7 in aqueous medium (Courtney, 1980, and our Experimental Procedures). However, an important difference is that tetracaine is a more hydropho-

bic and membrane-soluble compound than procaine, as indicated by an oleylalcohol/water partition coefficient of 80 as compared with 0.6 for procaine (Covino and Vassallo, 1976). QX314 is a quaternary amine with a very low oleylalcohol/water partition coefficient (Hille, 1977).

Fig. 1 illustrates the effects of tetracaine on the time course of $^{45}Ca^{2+}$ release from heavy SR Ca^{2+} release vesicles. The vesicles were initially passively loaded with 1 mM $^{45}Ca^{2+}$ and then diluted into release media that either inhibited or partially activated the SR Ca^{2+} release channel. As previously observed (Meissner, 1984), $^{45}Ca^{2+}$ release was slow in a medium containing the two Ca^{2+} release inhibitors Mg^{2+} and ruthenium red. Addition of 500 μM tetracaine to the Ca^{2+} release-inhibiting medium did not appreciably affect the $^{45}Ca^{2+}$ efflux behavior of the vesicles (not shown). When vesicles were placed in a medium containing 10 μM free Ca^{2+}, which partially

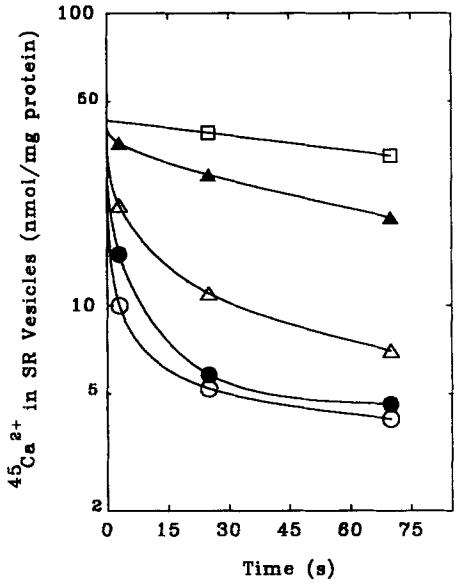

FIGURE 1. Tetracaine inhibition of $^{45}Ca^{2+}$ efflux from heavy SR vesicles. SR vesicles were passively loaded with 1 mM $^{45}Ca^{2+}$ and diluted 200-fold into release media containing 20 mM KPipes, pH 7, 0.1 M KCl, and either 10 mM Mg^{2+}, 10 μM ruthenium red, and 1 mM EGTA (□), or 10 μM free Ca^{2+} (100 μM EGTA and 105 μM Ca^{2+}) (○) plus 25 (●), 100 (△), or 500 (▲) μM tetracaine. $^{45}Ca^{2+}$ efflux rates were determined as described in Experimental Procedures. Data from one representative experiment are shown.

activates the Ca^{2+} release channel (Smith et al., 1986), a majority of vesicles released their $^{45}Ca^{2+}$ contents in <30 s. Some radioactivity remained with the vesicles for longer times, indicating the presence of a small subpopulation of vesicles lacking the Ca^{2+} release channel (Meissner, 1984). Addition of 25 and 100 μM tetracaine to the 10 μM Ca^{2+} medium noticeably reduced the $^{45}Ca^{2+}$ efflux rate. At 500 μM tetracaine, $^{45}Ca^{2+}$ efflux rates approached those measured in the presence of 10 mM Mg^{2+} and 10 μM ruthenium red. Accordingly, elevated concentrations of tetracaine appeared to almost fully inhibit the Ca^{2+}-activated SR Ca^{2+} release channel.

The rate of $^{45}Ca^{2+}$ efflux from heavy SR vesicles is dependent on the composition of the release medium, such as free Ca^{2+}, Mg^{2+}, and adenine nucleotide concentration, or pH (Meissner, 1984; Meissner, Darling, and Eveleth, 1986). Fig. 2 shows that dilution of vesicles into pH 7 release media containing either 125 μM free Ca^{2+} or 20

μM free Ca^{2+}, 1 mM Mg^{2+}, and 5 mM AMP yielded half-times of $^{45}Ca^{2+}$ release of 10 and 11 s, respectively. In both release media, tetracaine was similarly effective in inhibiting $^{45}Ca^{2+}$ efflux. Half-times of $^{45}Ca^{2+}$ efflux increased to ~20 and 100 s in the presence of 50 and 500 μM tetracaine, respectively. A nearly complete inhibition of $^{45}Ca^{2+}$ release by 500 μM tetracaine was also observed when the Ca^{2+} release channel was activated by placing vesicles into release media containing $< 10^{-8}$ M free Ca^{2+} plus 2.5 mM ATP or 10 μM free Ca^{2+}, 8 mM Mg^{2+}, and 6 mM adenosine 5'(β,γ-methylene)triphosphate (AMP-PCP) (not shown).

Tetracaine is a tertiary amine (pKa 8.5 in 0.1 M KCl; see Experimental Procedures) that is ~97% protonated at pH 7. The inhibitory effects of the protonated and uncharged forms of tetracaine were tested by measuring $^{45}Ca^{2+}$ efflux at pH 7.8 and 6.8, which changes the extent of protonation to 80 and 98%, respectively. Vesicles were diluted into release media containing 20 μM free Ca^{2+}, 5 mM AMP, and millimolar Mg^{2+}. The concentration of Mg^{2+} was adjusted to 5 mM (at pH 7.8) and 1

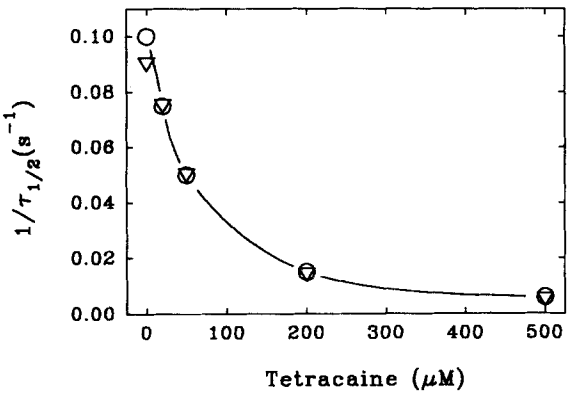

FIGURE 2. Dependence of $^{45}Ca^{2+}$ efflux on tetracaine concentration. SR vesicles were passively loaded with 5 mM $^{45}Ca^{2+}$ and diluted 100-fold into release media containing 20 mM KPipes, pH 7, 0.1 M KCl, the indicated concentrations of tetracaine, and either 125 μM free Ca^{2+} (100 μM EGTA, 225 μM Ca^{2+}) (○) or 20 μM free Ca^{2+} (100 μM EGTA, 115 μM Ca^{2+}) plus 5 mM AMP and 1 mM Mg^{2+} (▽). The $^{45}Ca^{2+}$ efflux half-times of the Ca^{2+}-permeable vesicle fraction are shown. Data of two representative experiments are shown.

mM (at pH 6.8) to obtain similar half-times of $^{45}Ca^{2+}$ efflux (10 and 15 s, respectively). In both release media, the addition of 100 μM tetracaine nearly doubled the half-time of $^{45}Ca^{2+}$ efflux (not shown). This result suggests that the efficacy of tetracaine in inhibiting SR Ca^{2+} release activity was not appreciably altered by a 10-fold change in the concentration of the uncharged form of tetracaine (20 and 2 μM at pH 7.8 and 6.8, respectively).

The effects of procaine and QX314 on the $^{45}Ca^{2+}$ release behavior of SR vesicles were studied following the procedure described in Fig. 1 by diluting vesicles into release media containing 10 μM free Ca^{2+} and millimolar concentrations of the two compounds. The half-time of $^{45}Ca^{2+}$ efflux was doubled in the presence of 2.5 ± 1.0 mM procaine (n = 3; Table I). A similar efficacy of procaine inhibition was observed in release media that contained $< 10^{-8}$ M free Ca^{2+} plus 0.5 mM ATP or 10 or 100 μM free Ca^{2+}. This result suggested that the effectiveness of procaine in inhibiting the SR Ca^{2+} release channel was not significantly altered when the channel was

activated by either ATP (Meissner et al., 1986) or Ca^{2+}. Preincubation of vesicles with 1–10 mM procaine for 2 h at 23°C, followed by dilution into release media containing the same procaine concentration, did not increase the efficacy of procaine inhibition. In other experiments, vesicles were incubated with 10 mM procaine or 500 μM tetracaine and local anesthetics were then removed by washing of vesicles before passive loading with 1 mM $^{45}Ca^{2+}$. Restoration of full $^{45}Ca^{2+}$ release activity

TABLE I

Kinetic Parameters of SR Ca^{2+} Release Channel Inhibition and Blockade

Parameter	Tetracaine	Procaine	QX314
Inhibition of $^{45}Ca^{2+}$ efflux			
L_{50} (mM)	0.10 ± 0.05 (n = 4)	2.5 ± 1.0 (n = 3)	Not observed
Inhibition of single channel activity			
L_{50} (mM)	0.15 ± 0.05 (n = 11) 0.6 ± 0.2 (n = 8)	4.0 ± 2.5 (n = 14) > 10 (n = 4)	Not observed
Single channel blockade			
− Ryanodine			
k_{bo} (mM^{-1} ms^{-1})			4.4 ± 0.2
V_{bo} (mV)			−72.4 ± 6.4
k_{-bo} (ms^{-1})			54.4 ± 4.0
V_{-bo} (mV)			28.8 ± 2.8
K_{Do} (mM)			13.2 ± 0.7
V_{Do} (mV)			19.7 ± 1.1
+ Ryanodine			
k_{bo} (mM^{-1} ms^{-1})		0.83 ± 0.14	0.13 ± 0.01
V_{bo} (mV)		−35.6 ± 4.2	−32.0 ± 2.1
k_{-bo} (ms^{-1})		27.7 ± 8.9	7.2 ± 0.5
V_{-bo} (mV)		−197 ± 288	24.7 ± 1.5
K_{Do} (mM)		33.4 ± 0.1	55.4 ± 6.2
V_{Do} (mV)		43.4 ± 0.1	14.2 ± 3.3

L_{50} indicates the local anesthetic concentration that resulted in half-maximal reduction of $^{45}Ca^{2+}$ efflux rates and channel open probabilities. Derived parameters of single channel blockade were obtained from continuous recordings by analysis of current amplitude distributions of local anesthetics as described in the text. Closed channel contributions to current amplitude histograms were removed as described by Yellen (1984) and Quayle et al. (1988).

suggested that the two local anesthetics inhibited SR Ca^{2+} release in a reversible manner. Addition of 5 mM QX314 to the 10 μM Ca^{2+} release medium did not result in a noticeable inhibition of $^{45}Ca^{2+}$ release regardless of whether vesicles were passively loaded with $^{45}Ca^{2+}$ for 2 h in the presence or absence of 5 mM QX314. These results suggest that the order of effectiveness in inhibiting Ca^{2+} release from heavy SR vesicles was tetracaine > procaine > QX314.

Single Channel Measurements with Tetracaine

The effects of tetracaine on the SR Ca^{2+} release channel were also more directly visualized by incorporating purified, CHAPS-solubilized channels into Mueller-Rudin planar lipid bilayers. The reconstituted SR Ca^{2+} release channel does not conduct anions such as Cl^-, and it conducts monovalent cations more efficiently than it does Ca^{2+} (Lai et al., 1988; Smith et al., 1988). Accordingly, channel activity can be conveniently monitored in a monovalent cation medium. In a majority of our recordings, single channel activity was greatly reduced and often irreversibly lost when either positive or negative membrane potentials of magnitude >60 mV were applied to the bilayers for several minutes. The effects of tetracaine on single channel activity were therefore examined only in the relatively narrow voltage range of −60 to +60 mV. For membrane potentials ranging from −20 to +20 mV, tetracaine inhibition of channel activity was largely independent of holding potential. Outside of this range, variable effects were observed. In some recordings, tetracaine nearly equally inhibited channel activity for both negative and positive membrane potentials, whereas in other experiments the inhibitory action was more evident on one side of the reversal potential (E_{rev}) than on the other side.

The purified channels were inserted into the bilayers in the presence of a symmetric 0.25 M KCl, 20 mM KPipes, pH 7 medium (Fig. 3). In the upper trace of Fig. 3 *A*, the channel was activated by 100 μM Ca^{2+} in the *cis* chamber (the side of the

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Figure 3. (*opposite*) Effect of tetracaine on single channel activity of the purified Ca^{2+} release channel complex. (*A*) Single channel currents, shown as downward deflections (*c*, closed), were recorded in symmetrical 0.25 M KCl media containing 100 μM free Ca^{2+} *cis* (100 μM EGTA, 200 μM Ca^{2+}) and 6 μM free Ca^{2+} *trans* (100 μM EGTA, 100 μM Ca^{2+}). Holding potential = 10 mV. Bars beside each trace indicate the level of the closed state. (*Top trace*) In the absence of tetracaine, P_o = 0.55. (*Middle trace*) With 200 μM tetracaine *cis*, P_o = 0.21. (*Bottom trace*) With 500 μM tetracaine *cis*, P_o = 0.05. (*B*) Single channel currents, shown as downward deflections, in symmetrical 0.25 M KCl media containing 9 μM free Ca^{2+} plus 2.4 mM ATP *cis* and 50 μM free Ca^{2+} *trans*. HP = +19 mV. (*Top trace*) With no tetracaine, P_o = 0.88. (*Middle trace*) With 200 μM tetracaine *trans*, P_o = 0.38. (*Bottom trace*) With 500 μM tetracaine *trans*, P_o = 0.12. (*C*) Currents, shown as upward deflections, in symmetrical 0.25 M KCl media containing 50 μM free Ca^{2+} *cis* and *trans*. HP = −25 mV. (*Top trace*) Without tetracaine, P_o = 0.49. (*Middle trace*) With 200 μM tetracaine *trans*, P_o = 0.11. (*Bottom trace*) After perfusion of *trans* chamber with 0.25 M KCl medium containing 50 μM free Ca^{2+}, P_o = 0.48. (*D*) Diary plot of the experiment in *C*. Shown are values of P_o as fractional open times during successive sweeps of 250 ms. (*1*) Control without tetracaine (105 s), (*2*) after addition of 200 μM tetracaine *trans* (105 s), and (*3*) after perfusion (90 s). The two gaps between *1* and *2*, and *2* and *3* correspond to periods of stirring and perfusion, respectively. (*E*) Dependence of Ca^{2+} release channel activity on tetracaine concentration. Relative fractional channel open times (P_x/P_o) were obtained from single channel recordings similar to those shown in *A* (○), and *B* (△). Solid lines were obtained according to the equation

$$P_x/P_o = [1 + ([T]/K_D)^{n_H}]^{-1}$$

where T is the indicated tetracaine concentration, and using dissociation constants (K_D) of 170 μM (○) and 180 μM (△) and Hill coefficients (n_H) of 2.5 and 2.2, respectively.

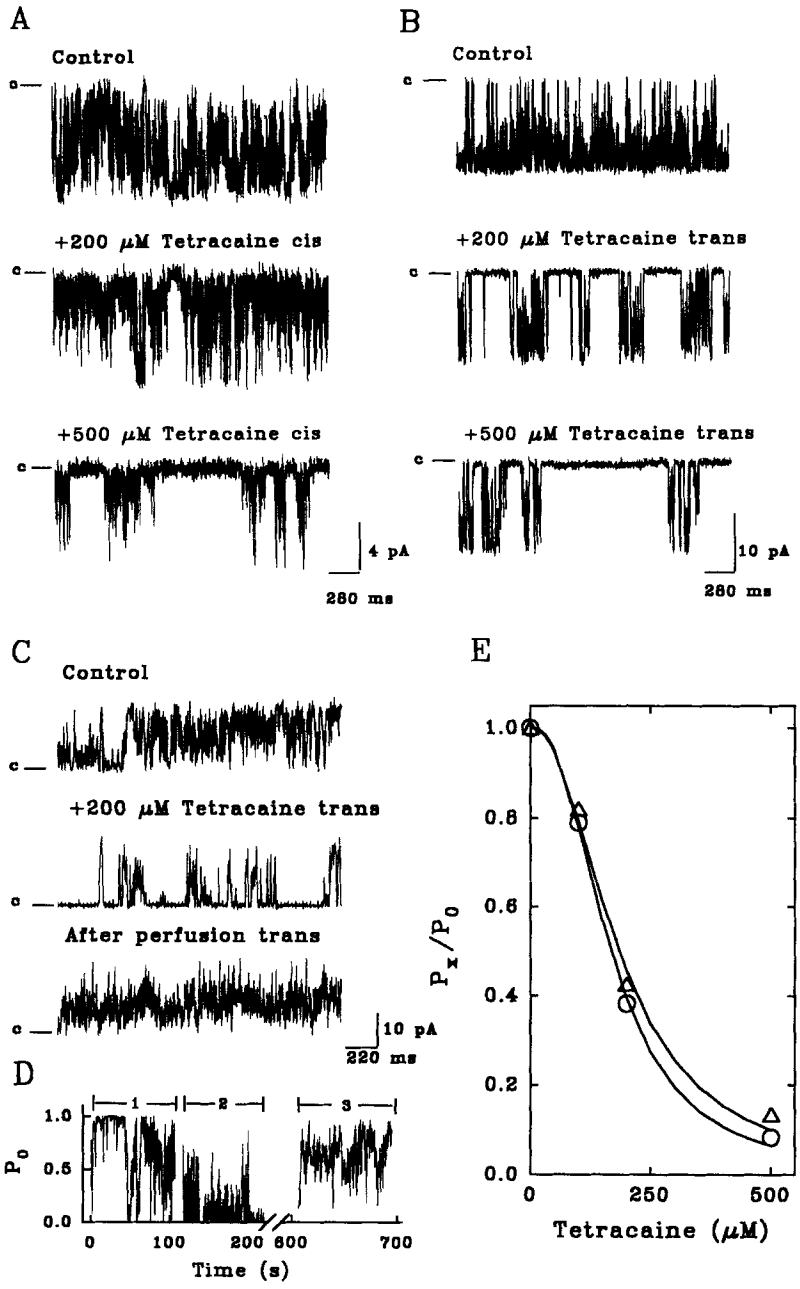
A
Control
c
+200 μM Tetracaine cis
+500 μM Tetracaine cis
4 pA
280 ms
B
Control
+200 μM Tetracaine trans
+500 μM Tetracaine trans
10 pA
280 ms
C
Control
+200 μM Tetracaine trans
After perfusion trans
10 pA
220 ms
D
1
2
3
P_o
1.0
0.5
0.0
0
100
200
600
700
Time (s)
E
P_x/P_o
1.0
0.8
0.6
0.4
0.2
0.0
0
250
500
Tetracaine (μM)

FIGURE 3.

bilayer to which the channel was added; *cis* and *trans* chambers correspond to the SR cytoplasmic and lumenal sides, respectively [Miller, 1978; Smith et al., 1986]). Channel activity was greatly reduced upon the addition of *cis* tetracaine. The drug reduced the P_o of the Ca^{2+}-activated channel from 0.55 to 0.21 and 0.05 at 200 and 500 μM tetracaine, respectively. At 500 μM, channel openings were inhibited for prolonged intervals without an apparent change in single channel conductance (772 pS in 0.25 M K^+; see below). Current traces of a Ca^{2+}- and ATP-activated Ca^{2+} release channel in Fig. 3 *B* show a similar inhibition of channel activity when tetracaine is added to the *trans*, rather than the *cis*, side of the bilayer. In the absence of tetracaine (upper trace of Fig. 3 *B*), the channel displayed prolonged periods of channel openings resulting in a nearly optimally activated channel. Channel openings were only occasionally interrupted by periods of silent channel activity lasting >50 ms. The addition of 200 and 500 μM tetracaine *trans* decreased channel open probability from 0.88 to 0.38 and 0.12, respectively. Again, tetracaine induced the formation of long closed channel intervals that were disrupted by periods of rapid channel openings and closings.

Reversibility of channel inhibition by tetracaine was tested in six experiments by recording channel activity before and after the addition of tetracaine, and after removal of the drug by perfusion. In four of these experiments, the bilayer broke during the prolonged (~7 min) perfusion step. In the two remaining experiments, one of which is shown in Fig. 3, *C* and *D*, a nearly complete restoration of channel activity was observed after removal of the drug. This result suggested that tetracaine reversibly inhibited the channel, as observed in $^{45}Ca^{2+}$ flux measurements.

Tetracaine concentrations needed for a twofold reduction of channel P_o ranged from 75 to 750 μM (n = 19). In 11 of 19 experiments, tetracaine concentrations comparable to those observed in vesicle–$^{45}Ca^{2+}$ flux experiments (Figs. 1 and 2) were effective in reducing channel P_o twofold, with a mean tetracaine concentration of (150 ± 50) μM (Fig. 3 *E*). A twofold decrease in P_o was observed with either 150 μM *cis* or *trans* tetracaine for channels that were activated by either micromolar Ca^{2+} (n = 6) (Fig. 3 *A*) or micromolar Ca^{2+} plus mM ATP (n = 4) (Fig. 3 *B*), or that were recorded in the presence of 50 μM free Ca^{2+} and millimolar ATP and Mg^{2+} *cis* (n = 1) (not shown). In other experiments (8 of 19), higher levels of tetracaine with a mean concentration of 600 ± 200 μM were required to effect a twofold reduction of channel P_o (not shown). An average Hill coefficient of 2.5 ± 0.4 (n = 3) (Fig. 3 *E*) suggests that the SR Ca^{2+} release channel contains interacting inhibitory binding sites for tetracaine.

In Fig. 4 *A*, the current–voltage relationships of a single channel, recorded in symmetric 0.25 M KCl, 20 mM KPipes, pH 7, buffer, are plotted in the presence and absence of tetracaine. A slope conductance of 772 ± 43 pS (n = 47) was obtained for the K^+ conducting release channel; the conductance remained essentially unchanged after the addition of 500 μM tetracaine *cis* (Fig. 4 *A*) or *trans* (not shown), or the addition of 500 μM or 1 mM tetracaine to both sides of the bilayer (not shown). The effects of tetracaine on single channel conductance were also tested in the presence of 10 mM Ca^{2+} *trans* (Fig. 4 *B*). Addition of 10 mM Ca^{2+} to the *trans* chamber significantly reduced single channel currents at negative holding potentials and, to a lesser extent, at positive voltages. The reversal potential (E_{rev}) was shifted to +5 mV, from which, by applying constant field theory, a permeability ratio of Ca^{2+} over K^+

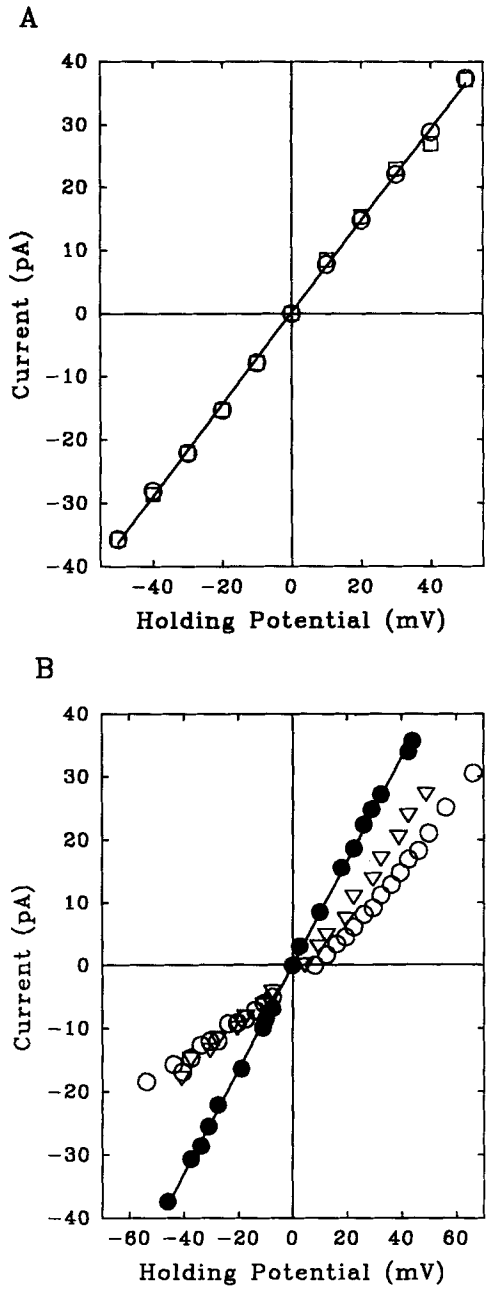

FIGURE 4. Current–voltage relationships of K^+-conducting Ca^{2+} release channel. (*A*) Single channel currents were recorded in symmetrical 0.25 M KCl media containing 9 μM free Ca^{2+} plus 2.4 mM ATP *cis* and 6 μM free Ca^{2+} *trans*, in the absence (○) and presence (□) of 500 μM tetracaine *cis*. Data from one representative experiment are shown. γ_{K^+} = 740 pS. (*B*) Currents were recorded in symmetrical 0.25 M KCl media containing 15 μM free Ca^{2+} plus 1.2 mM ATP *cis* and 50 μM (●) or 10 mM (▽) Ca^{2+} *trans*, and with 10 mM Ca^{2+} *trans* and 500 μM tetracaine *cis* and *trans* (○). Standard deviations were equal to or smaller than the size of the symbols (n = 4).

ions (P_{Ca}/P_K) of 3.8 was calculated, in accordance with previous measurements (Smith et al., 1988; Liu et al., 1989). Tetracaine augmented the effects of *trans* Ca^{2+} by shifting E_{rev} to slightly more positive values, suggesting a possible modification of the permeability behavior of the channel, and also by further reducing single channel current values, particularly at positive holding potentials. The effects on these

current values were essentially the same when tetracaine was added either to the *cis* or *trans* chamber or to both chambers simultaneously. The efficacy of tetracaine in decreasing channel P_o in the presence of 10 mM Ca^{2+} *trans* was comparable to that observed in the absence of millimolar Ca^{2+} *trans* (Fig. 3).

Single Channel Measurements with Procaine

In agreement with vesicle–$^{45}Ca^{2+}$ flux measurements, millimolar concentrations of procaine were required to noticeably reduce SR Ca^{2+} release channel activity. In Fig.

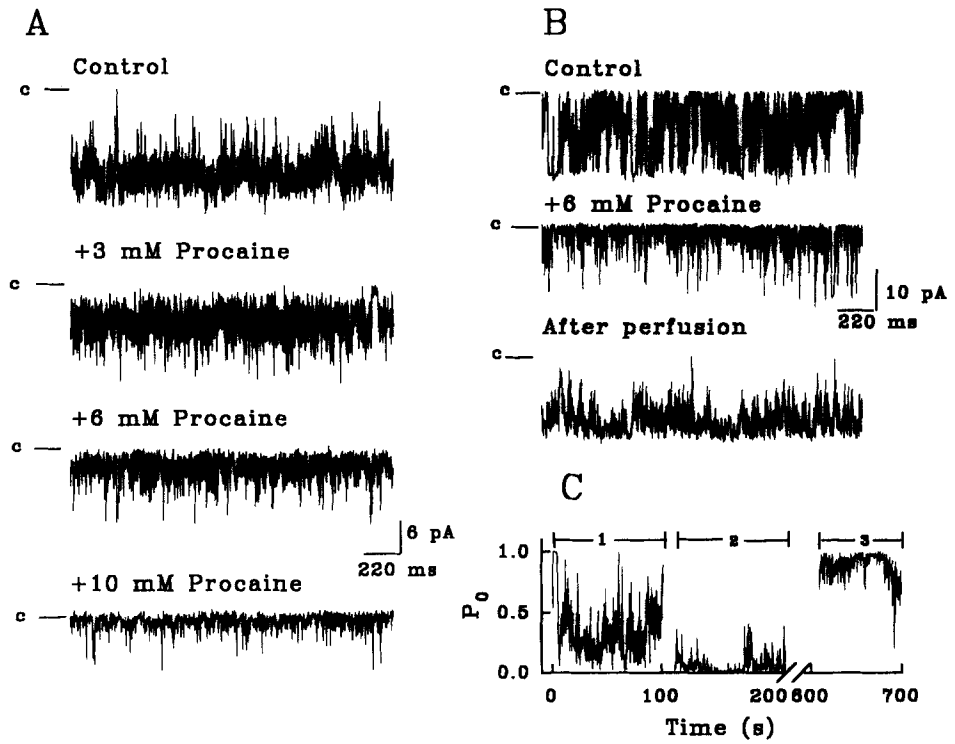

FIGURE 5. Effect of procaine on single channel activity of Ca^{2+} release channel. (*A*) Single channel currents, shown on the left as downward deflections (*c*, closed), were recorded in symmetrical 0.25 M KCl media containing 9 μM free Ca^{2+} plus 2.4 mM ATP *cis* and 50 μM free Ca^{2+} *trans*. Holding potential = 20 mV. (*Top trace*) Without procaine, P_o = 0.84. (*Second trace*) With 3 mM procaine *cis*, P_o = 0.33. (*Third trace*) With 6 mM procaine *cis*, P_o = 0.11. (*Fourth trace*) With 10 mM procaine *cis*, P_o = 0.03. (*B*) Currents, shown as downward deflections, in symmetrical 0.25 KCl media containing 9 μM free Ca^{2+} and 2.4 mM ATP *cis* and 50 μM free Ca^{2+} *trans*. Holding potential = 25 mV. (*Top trace*) Without procaine, P_o = 0.35. (*Middle trace*) With 6 mM procaine *cis*, P_o = 0.06. (*Bottom trace*) After perfusion of *cis* chamber for 10 min with 0.25 M KCl medium containing 9 μM free Ca^{2+} and 2.4 mM ATP, P_o = 0.86. (*C*) Diary plot Ca^{2+} of the experiment in B. Shown are values of P_o as fractional open times during successive sweeps of 250 ms. (*1*) Control before addition of procaine (105 s), (*2*) after addition of 6 mM procaine *cis* (105 s), and (*3*) after perfusion (90 s). The two gaps between *1* and *2*, and *2* and *3* correspond to periods of stirring and perfusion, respectively.

5 *A*, the open probability of a Ca^{2+}- and ATP-activated channel was reduced from 0.84 to 0.33, 0.11, and 0.03, respectively, by the addition of 3, 6, and 10 mM procaine to the *cis* chamber. A Hill coefficient of 2.2 ± 0.1 (n = 2) of procaine inhibition again suggested an allosteric inhibition of SR Ca^{2+} release channel activity by local anesthetics. In 14 of 18 experiments, procaine concentrations comparable to those obtained in vesicle–$^{45}Ca^{2+}$ flux studies (Table I) were effective in reducing P_o twofold, with a mean concentration of (4 ± 2.5) mM procaine. The decrease in P_o was observed with *cis* (12 of 14 experiments) and *trans* (2 of 4 experiments) procaine for channels that were activated by ~10 μM Ca^{2+} in the presence of 2–3 mM ATP (n = 12), or by 100 μM Ca^{2+} in the presence and absence of 5 mM AMP (n = 2). In six (of six) experiments procaine nearly equally reduced channel P_o at holding potentials of −20 and +20 mV. In the remaining experiments (4 of 18), procaine concentrations ranging from 5 to 15 mM were ineffective or only marginally effective in reducing channel P_o (not shown). In two of these, we observed a pronounced voltage-dependent reduction in apparent single channel currents; however, because of its infrequent occurrence this phenomenon was not further studied.

Reversibility of inhibition of single channel activity by procaine was tested in 11 experiments. In three of these, the bilayer broke during the perfusion step. In one experiment, a twofold reduction in the unitary conductance of the channel was observed after perfusion. In two experiments, no channel activity could be observed after perfusion. In the remaining five experiments, P_o either increased (n = 3) or decreased (n = 2) up to ~2.5-fold after perfusion when compared with P_o values in the control condition. Fig. 5, *B* and *C* show an experiment in which P_o increased after the perfusion step. Addition of 6 mM procaine *cis* reduced the P_o of the Ca^{2+}- and ATP-activated channel from 0.35 to 0.06. After removal of the drug by perfusion of the *cis* chamber with a 0.25 M KCl medium, P_o increased to 0.86.

Single Channel Recordings with QX314

The quaternary amine QX314 did not noticeably affect single channel conductance and P_o at all holding potentials when added to the *trans* chamber at concentrations ranging from 2 to 10 mM (not shown). In contrast, a pronounced voltage-dependent reduction in single channel currents was apparent at positive holding potentials in six of six experiments when QX314 was added to the *cis* chamber (Fig. 6). No major reduction in current amplitude was observed upon return to negative holding potentials. Reduction in current amplitude could be also fully reversed by perfusion of the *cis* chamber with a QX314-free solution (n = 1; not shown).

Block of Ryanodine-modified Ca^{2+} Release Channel

The neutral plant alkaloid ryanodine induces in single channel recordings a long-lived subconductance state of the SR Ca^{2+} release channel (upper trace of Fig. 7; Rousseau, Smith, and Meissner, 1987; Smith et al., 1988; Liu et al., 1989). The ryanodine-modified channel state is largely insensitive to subsequent addition of *cis* Ca^{2+}, ATP, or Mg^{2+}, physiological regulators that otherwise greatly affect the gating behavior of the channel. Ryanodine modification also rendered the channel less sensitive to inhibition by tetracaine, since addition of 1 mM tetracaine to both sides of

the bilayer reduced channel P_o only minimally at negative holding potentials (from ~1 to 0.97–0.99, $n = 4$; not shown). At positive holding potentials, tetracaine was somewhat more effective in causing partial channel closing. At +60 mV, P_o was lowered from ~1 to values ranging from 0.97 to 0.55 ($n = 4$). A second effect of tetracaine was to reduce single channel conductances at positive holding potentials in a voltage-dependent manner. In the presence of 1 mM tetracaine on both sides of the bilayer, the current values were reduced at +60 mV by 25 ± 13% ($n = 7$; second and third traces of Fig. 7). A similar reduction was observed when 2 mM tetracaine was added to either side of the bilayer (not shown), indicating that the effect was caused

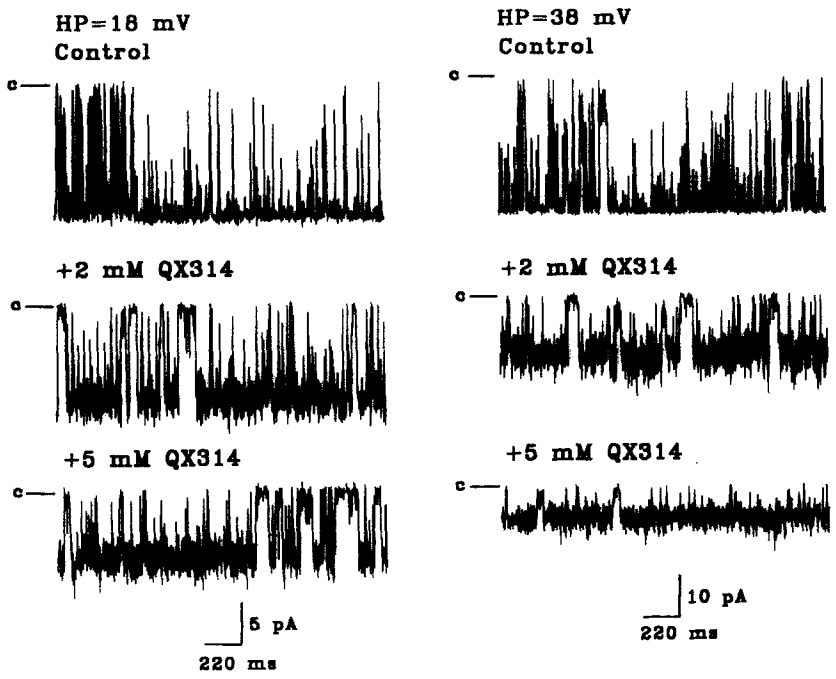

FIGURE 6. Single channel recordings in the presence of QX314. Single channel currents were recorded in symmetrical 0.25 M KCl media containing 16.5 μM free Ca^{2+}, 2.4 mM ATP, 0, 2, or 5 mM QX314 *cis*, and 100 μM free Ca^{2+} *trans*. Holding potential = 18 (*left traces*) and 38 (*right traces*) mV.

by the total concentration of the drug that was present in the system. At negative holding potentials, the current values of the ryanodine-modified channel were not altered by the presence of up to 2 mM tetracaine in either the *cis* or *trans* chamber.

We also found that *cis* procaine reduced the conductance of the ryanodine-modified channel (fourth trace of Fig. 7; $n = 3$). This block was only observed at positive voltages and was voltage dependent (not shown). Addition of QX314 to the *cis* chamber induced a flickery block of the ryanodine-modified channel (bottom trace of Fig. 7; $n = 3$). As observed for the ryanodine-unmodified channel, addition of QX314 to the *trans* chamber did not affect the gating and conductance of the ryanodine-modified channel at negative or positive holding potentials ($n = 3$).

Analysis of Block of Ca^{2+} Release Channel by Local Anesthetics

Experiments with QX314 (Fig. 6) and the ryanodine-modified channel (Fig. 7) indicated that local anesthetics may inhibit SR Ca^{2+} release by a direct blocking mechanism. The interaction of a blocking ion (B) with the open state of the channel can be illustrated by the following reaction:

$$R_o + B \underset{k_{-b}}{\overset{k_b}{\rightleftharpoons}} R_B$$

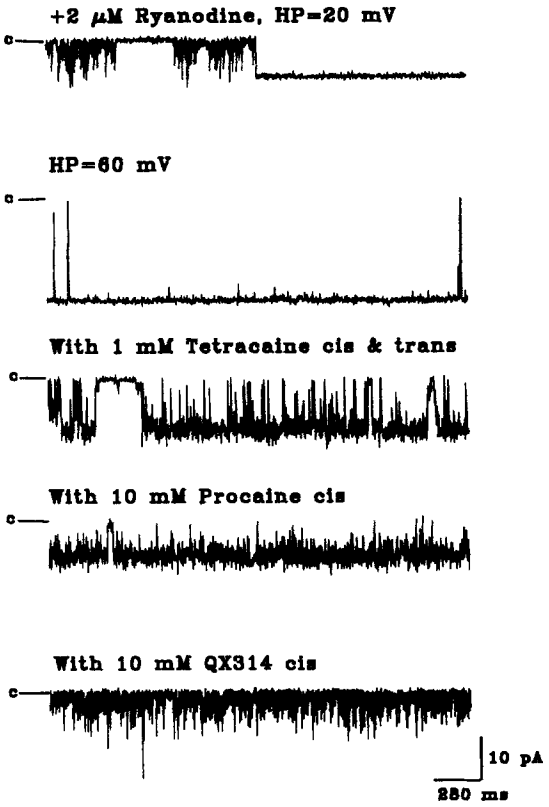

FIGURE 7. Effects of local anesthetics on the ryanodine-modified SR Ca^{2+} release channel. Single channel currents were recorded in symmetrical 0.25 M KCl media containing 50 μM free Ca^{2+}. The top trace shows a sudden change in the gating and conductance behavior of the channel ~4 min after the addition of 2 μM ryanodine *cis*. P_o increased from 0.1 to nearly 1, whereas single channel conductance decreased from 750 to 400 pS. Also shown are single channel recordings of the ryanodine-modified channel at +60 mV before (*second trace*) and after the addition of 1 mM tetracaine *cis* and *trans* (*third trace*), 10 mM procaine *cis* (*fourth trace*), and 10 mM QX314 *cis* (*fifth trace*). Traces *1–3*, *4*, and *5* show three separate experiments.

where R_o is the open channel state, R_B is the blocked channel state, and k_b and k_{-b} are the blocking and unblocking rates, respectively. The appearance of single channel recordings produced by the action of channel blockers depends on the length of time that the channel is in state R_B and the time resolution of the recording system (Yellen, 1984). If the action of the channel blocker is very fast, such that the duration of the blocking events is too short to be resolved, only the time-averaged current between the open (R_o) and the blocked (R_B) states is measured, leading to an apparent reduction in the recorded channel current. Such an action is considered to be a fast blocking process. A slow blocking process, on the other hand, contains

blocking events that last long enough to be fairly well resolved by the recording system. Finally, an intermediate or flickery blocker produces events long enough to be detected by the system but not long enough to be well resolved. Current amplitude histograms produced by the action of fast blockers show a single sharp peak corresponding to the time-averaged current, while those generated by slow blockers show two clearly resolved peaks corresponding to the current amplitudes of

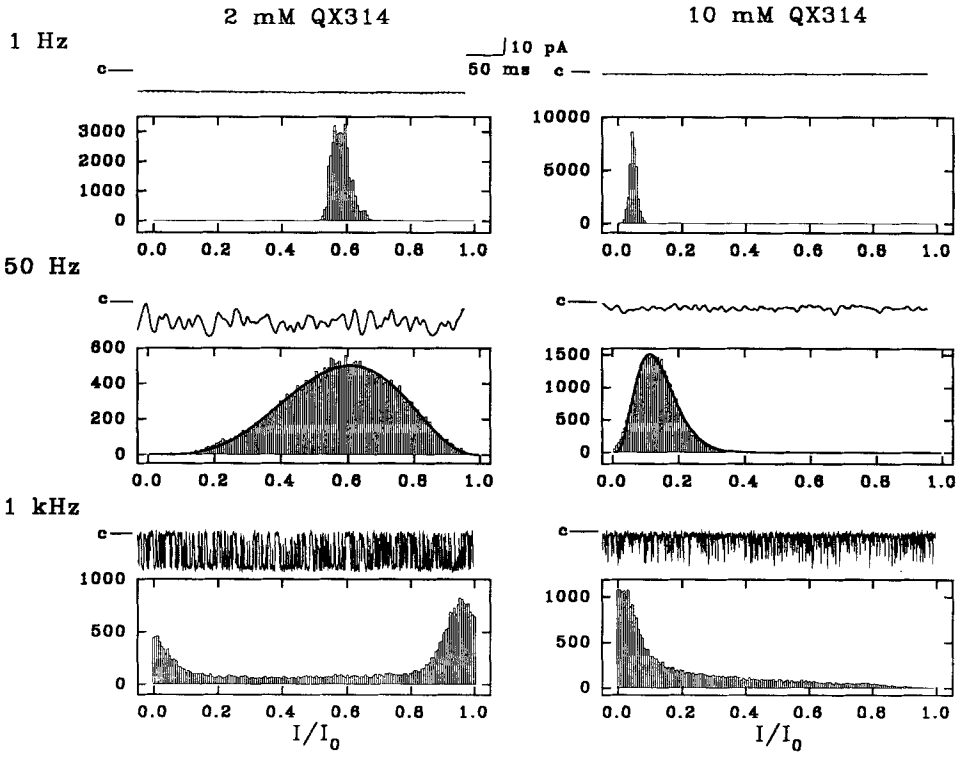

FIGURE 8. Current amplitude histograms of ryanodine-modified channel in the presence of 2 and 10 mM QX314. Current amplitude histograms were constructed from recordings (top of each panel) at a low (1 Hz), intermediate (50 Hz), and high (1,000 Hz) bandwidth of the recording system. Holding potential = +47 mV. Current in the absence of QX314 (I_o) = 21.0 pA. Ordinate gives number of occurrences per 17 s (at 1 and 50 Hz) and 7 s (at 1 kHz) files. Abscissa represents the relative current amplitude, with 0 and 1 corresponding to baseline current and open channel amplitude without QX314, respectively. The solid lines in the two middle panels were calculated according to Eq. 2 and using the following model parameters at 2 and 10 mM QX314 *cis*, respectively: a = 4.83 and 4.05, b = 3.47 and 26.04.

the blocked and the unblocked (open) channels (Yellen, 1984). Intermediate blocking processes yield broad current amplitude distributions; if the blocking and unblocking rates are markedly different, these distributions may be skewed (Yellen, 1984).

The blocking action of QX314 on the ryanodine-modified channel was analyzed as a fast, intermediate, or slow blocking process by varying the filter setting to change the bandwidth of the recording system. Fig. 8 shows the apparent current recordings

and current amplitude histograms produced by the ryanodine-modified channel in the presence of 2 and 10 mM QX314 *cis* at filter settings of 1, 50, and 1,000 Hz. At 1 Hz, no discrete channel transitions were observed. The current amplitude distributions were narrow with a single peak characteristic of a fast blocking process (upper two panels). Such a channel blockade can be described by

$$\frac{I_B}{I_o} = \frac{1}{1 + [B]/K_D} \tag{1}$$

where I_B is the time-averaged single channel current produced by the blocking process, I_o is the current recorded in the absence of the blocking ion, [B] is the concentration of the blocking ion, and K_D is the dissociation constant. Values of I_B

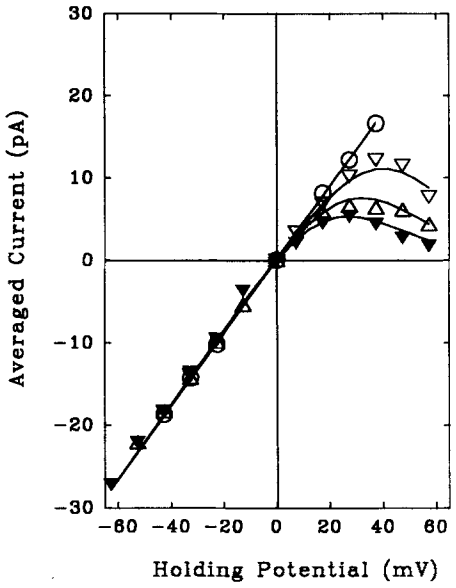

FIGURE 9. Current–voltage relations of ryanodine-modified channel in the presence of QX314. Shown are the apparent current–voltage relations of K^+-conducting, ryanodine-modified channel in the absence (○) and presence of 2 (▽), 5 (△), and 10 mM (▼) QX314 *cis*. Filter = 1 Hz. Data were fitted according to Eq. 1 using derived parameters shown in Table I.

measured in the presence of QX314 varied in a concentration- and voltage-dependent manner (Fig. 9), indicating that the derived K_D values were voltage dependent at positive holding potentials (see below).

At a filter setting of 50 Hz, indistinct channel transitions were apparent (middle two panels of Fig. 8). The current amplitude distributions were broadened, indicative of an intermediate blocking process. These amplitude histograms were well fitted by distributions described by the following probability distribution function (Yellen, 1984):

$$f(y) = y^{a-1}(1 - y)^{b-1}/B(a, b) \tag{2}$$

where y is the current amplitude of the filter output, a ($= \alpha\tau$) and b ($= \beta\tau$) are model parameters dependent on τ, the effective time constant of the filter at the given frequency setting, and $B(a, b)$ is a beta function defined by

$$B(a, b) = \int_0^1 y^{a-1}(1 - y)^{b-1}\, dy \tag{3}$$

The blocking (k_b) and unblocking (k_{-b}) rates and the dissociation constant (K_D) were obtained from the parameters α and β using

$$k_b = \beta/[B] \tag{4}$$

$$k_{-b} = \alpha \tag{5}$$

$$K_D = k_{-b}/k_b \tag{6}$$

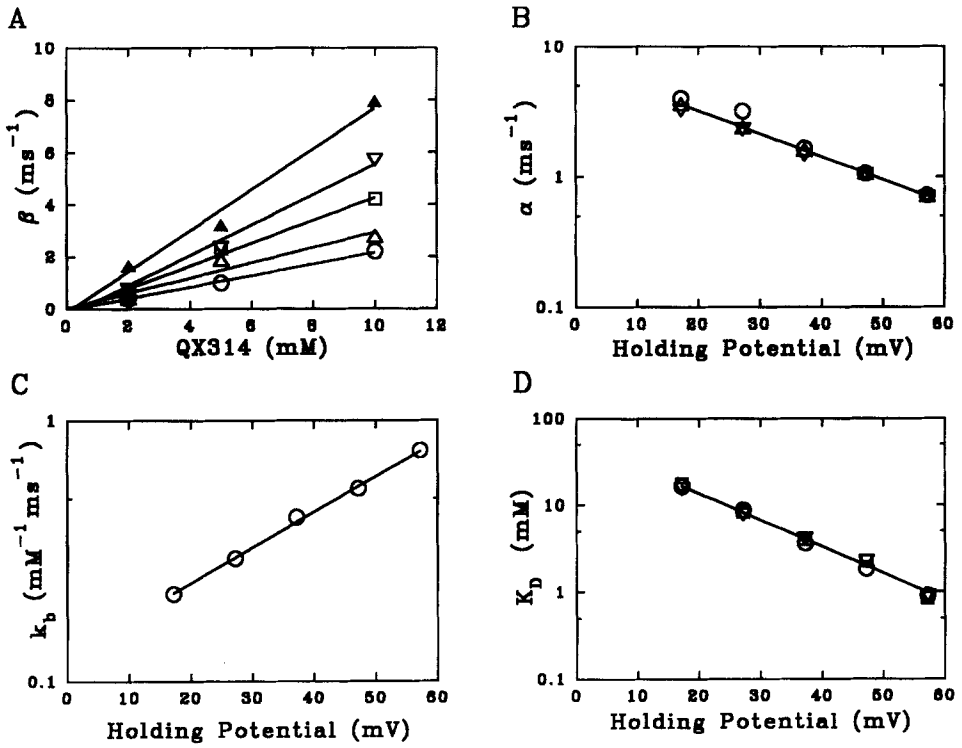

FIGURE 10. Derived parameters of analysis of QX314 blockade of ryanodine-modified channel. Blocking (β, k_b) and unblocking (α) parameters were obtained from a fitted beta function (Fig. 8) using an effective time constant (τ) of $0.228/f$, where f is 3-db attentuation frequency of the Bessel filter. In *A*, holding potentials were 17 (○), 27 (△), 37 (□), 47 (▽), and 57 (▲) mV. In *B*, *cis* QX314 concentrations were 2 (○), 5 (▽), and 10 (△) mM. In *D*, dissociation constants were obtained from recordings filtered at 1 (▽), 50 (○), and 1,000 (□) Hz, as described in the text. Standard deviations were equal to or smaller than symbols.

Values of a = 4.83 and 4.05 and b = 3.47 and 26.04 were obtained at 2 and 10 mM QX314 *cis*, respectively, by fitting Eqs. 2 and 3 to the amplitude distributions of the middle panels of Fig. 8 using the nonlinear least-squares routines. At holding potentials ranging from +17 to +57 mV, the blocking parameter β increased linearly with QX314 concentration (Fig. 10 *A*), whereas the unblocking parameter α was independent of [QX314] (Fig. 10 *B*). Both parameters, as well as the derived values of k_b, k_{-b}, and K_D, were voltage dependent at positive holding potentials, varying

linearly with the voltage in the semilogarithmic plots of Fig. 10, *B–D*. Such a voltage dependence can be described by an equation of the form

$$X = X_o \exp(-V/V_{xo}) \tag{7}$$

where X represents the given parameter, X_o is the apparent zero-voltage value of the parameter, and V_{xo} is a quantity associated with the parameter that describes the voltage dependence of the block. The derived values of k_{bo}, k_{-bo}, K_{Do}, V_{bo}, V_{-bo}, and V_{Do} for the QX314 block of the ryanodine-modified channel are listed in Table I. Fig. 10 *D* shows that essentially identical K_D/voltage relations were obtained when QX314 was analyzed as a fast (at 1 Hz), intermediate (at 50 Hz), or slow (at 1,000 Hz, see below) blocker.

At 1,000 Hz, the current amplitude histogram produced by the ryanodine-modified channel in the presence of 2 mM QX314 contained peaks corresponding to the current amplitudes of the blocked and unblocked (open) channel (lower left panel of Fig. 8), indicating that the block could be analyzed as a slow blocking process at this frequency. Such a blocking process can be analyzed using the following equation:

$$\frac{P_B}{P_o} = \frac{1}{1 + [B]/K_D} \tag{8}$$

where P_B and P_o are the channel open probability in the presence and absence of the blocker, respectively, and [B] and K_D have the same meaning as in Eq. 1. Channel P_o's were determined using a single threshold level set at 50% of the current amplitude obtained in the absence of QX314. Under these conditions, the K_D values exhibited a voltage dependence of the form described in Eq. 7; the derived K_D/voltage relation was essentially identical to those obtained by analyzing QX314 block as a fast and as an intermediate blocking process (Fig. 10 *D*; Table I). At 10 mM QX314, blockage was still too fast to yield fully resolved unblocked channel events at 1,000 Hz (lower right panel of Fig. 8).

The blocking actions of QX314 on the ryanodine-unmodified channel (Fig. 6) and of procaine on the ryanodine-modified channel (Fig. 7) were also analyzed as intermediate processes by fitting measured amplitude histograms to a beta function (Table I). Since blocking and unblocking rates were appreciably faster than those observed for the QX314 block of the ryanodine-modified channel (Fig. 7), single channel currents were analyzed at a filter setting of 1,000 Hz. Analysis of procaine as a fast blocker of the ryanodine-modified channel at a filter setting of 10 Hz yielded V_{Do} values that were essentially identical to, and K_{Do} values that differed by no more than a factor of two from, the values obtained in the intermediate blocking analysis listed in Table I. The voltage-dependent block of the ryanodine-modified channel by tetracaine could be described as an intermediate blocking process since at 1 kHz the current histograms could be fitted to a beta function. However, a strong dependence of the unblocking parameter α on tetracaine concentration, and a nonlinear dependence of the blocking parameter β on tetracaine concentration indicated that tetracaine did not block the ryanodine-modified channel by a simple blocking mechanism.

DISCUSSION

Previous studies have suggested that the local anesthetics tetracaine and procaine inhibit the release of Ca^{2+} ions from SR. The $^{45}Ca^{2+}$ release flux measurements presented here confirm the inhibitory effects of tetracaine and procaine on SR $^{45}Ca^{2+}$ release. The efficacy of tetracaine and procaine in inhibiting SR $^{45}Ca^{2+}$ release correlated with their oleylalcohol/water partition coefficients. While $^{45}Ca^{2+}$ efflux rates from passively loaded SR vesicles in the presence of 500 μM tetracaine approached those measured in the presence of the SR Ca^{2+} release channel inhibitors Mg^{2+} and ruthenium red, procaine concentrations in excess of 10 mM were required to nearly fully inhibit SR $^{45}Ca^{2+}$ release. The efficacy of this inhibition by tetracaine and procaine was similar under different channel activating conditions, and was not appreciably altered by a 10-fold change in the concentration of the uncharged form of tetracaine. QX314, a highly water-soluble, quaternary amine derivative of lidocaine, had no noticeable effect on vesicle flux measurements at a concentration of 5 mM.

Single channel measurements provided more detailed insights into the effects of local anesthetics on the SR Ca^{2+} release channel. We found that the channel can be inhibited by two different mechanisms as exemplified by the action of the lipid-soluble, tertiary amine tetracaine and the water-soluble, quaternary amine QX314 on the ryanodine-unmodified and -modified channel, respectively. The results of this study can be readily explained by postulating that the two drugs inhibited SR Ca^{2+} release by binding to two or more high-affinity regulatory sites located in or near the SR membrane and one low-affinity blocking site within the conduction pathway of the channel, respectively. The presence of multiple drug binding sites has been also postulated for the tetrodotoxin-sensitive Na^+ channel (Khodorov, Shishkova, Peganov, and Revenko, 1976; Moczydlowski, Uehara, and Hall, 1986; but see also Hille, 1977).

Allosteric Inhibition of Ryanodine-unmodified Channel by Tetracaine and Procaine

Inhibition of Ca^{2+}-gated channel activity by tetracaine and procaine was characterized by a lack of voltage dependence and membrane sidedness. These two observations are difficult to explain by a traditional blocking mechanism and instead suggest that the two local anesthetics inhibited channel activity by binding to a regulatory site that resides in or close to the bilayer. A Hill coefficient of ~ 2.5 suggested the presence of two or more cooperatively interacting drug binding sites whose occupation resulted in an allosteric modulation of the channel's structure and function.

In some experiments, lower tetracaine and procaine concentrations were ineffective in appreciably inhibiting channel activity. One possible explanation is a decreased binding affinity of the regulatory sites for local anesthetics in some of the channels, which may have occurred during purification and reconstitution. We do not believe that channels with a lower affinity for tetracaine or procaine are typical of SR channels since the efficacy of tetracaine and procaine inhibition in vesicle $^{45}Ca^{2+}$ flux experiments matched that of the higher, and not lower, affinity channels.

The effects of tetracaine on the Ca^{2+} release channel were more complex in the presence of millimolar Ca^{2+} on the *trans* side of the bilayer. We found that, in

addition to the gating, the conductance of the channel was modified. In the presence of 10 mM Ca^{2+} *trans* but in the absence of tetracaine, the reversal potential was shifted to positive values and the K^+ conductance of the channel was decreased. Reduction of K^+ conductance was greater at negative than at positive holding potentials. Since at negative voltages the current-carrying cations (K^+, Ca^{2+}) move from the *trans* (SR lumenal) side to the *cis* (SR cytoplasmic) side, this suggests that Ca^{2+} reduced the rate of K^+ passage through the channel by interacting more strongly than K^+ with site(s) within the ion conductance pathway. The addition of tetracaine to either side of the bilayer reduced P_o and shifted E_{rev} to more positive values, resulting in an increase of the calculated permeability ratio $P_{Ca^{2+}}/P_{K^+}$ from 3.8 to 6.8. Single channel current values were reduced more for positive than for negative voltages, partially offsetting the asymmetry in conductance induced by *trans* millimolar Ca^{2+}. Further experiments are needed to ascertain the properties of the asymmetric block of K^+ conductance by Ca^{2+} before modification of this block by tetracaine can be studied in more detail.

Inhibition of Ca^{2+}-gated Ca^{2+} release channel activity by procaine resembled that of tetracaine; however, several quantitative differences were noted. One was that in a majority of bilayer experiments a much higher concentration ($\sim$25 times greater) was needed for procaine to reduce channel P_o twofold (Table I). Second, procaine rarely induced the formation of very long closed events ($\tau > 200$ ms). Consequently, long closed intervals, interspersed by bursts of rapid channel activity, were only infrequently seen in the presence of procaine. Third, procaine occasionally induced a fast-blocking effect on the conductance of the ryanodine-unmodified channel without an apparent change in channel open time. Some of the differences in the action of procaine and tetracaine on SR Ca^{2+} release channel activity may be related to the more hydrophobic character of tetracaine. Although steric considerations may explain the differences in the action of the two local anesthetics, it is conceivable that tetracaine, by binding with higher affinity, dissociates less readily from hydrophobic binding sites, thereby inducing more substantial alterations in channel structure and function.

Single Channel Blockade by QX314

In contrast to tetracaine and procaine, QX314 did not inhibit SR Ca^{2+} release channel activity by an allosteric mechanism. Rather, effects of QX314 could be described by a traditional blocking mechanism in that single channel blockade was dependent on drug concentration and membrane potential, and was only observed when QX314 was added to the *cis* (SR cytoplasmic) side of the bilayer. As predicted from a simple theory of channel blockade (Woodhull, 1973), blocking and unblocking rates of QX314 had an opposite voltage dependence, although of different magnitude, and thereby both contributed to the voltage dependence of the dissociation constant of QX314 binding. Dissociation constants and their voltage dependences were obtained by analyzing current records of single ryanodine-modified channels at three filter settings. Essentially identical K_D values and voltage dependences were obtained, increasing our confidence in our data analysis. One interpretation of empirically derived voltage parameters has been provided by the single ion blocking model of Woodhull (1973). Applying the derived equation $V_{Do} = RT/z\delta F$, where δ is

the fraction of the membrane field felt by the blocking ion and R, T, z, and F have their usual meanings, and using V_{Do} values of 19.7 and 14.2 mV (Table I), we calculated that the blocking reaction of QX314 with the ryanodine-unmodified and -modified channel had effective valencies ($z\delta$) of 1.3 and 1.8, respectively. An effective valency of greater than one for a monovalent cation implies interaction of blocking ion with other ions present in the channel (Hille and Schwarz, 1978). In support of a multi-ion pore model that contains more than one ion at a time, the skeletal muscle SR Ca^{2+} release channel has been suggested to have a rather complex conductance pathway. The channel complex has an overall projected dimension of 27 × 27 × 14 nm and has been proposed to contain a central channel that connects to four radial channels located in the foot (cytoplasmic) region of the complex (Wagenknecht, Grassucci, Frank, Saito, Inui, and Fleischer, 1989). The data of this study are most readily explained by assuming that QX314 binds to a site located in a central, ion-conducting pore that is located in the membrane's electric field. In support of this suggestion, we found that at low QX314 concentrations the current fluctuations of the ryanodine-modified channel could be well resolved into open and closed events (Fig. 8) and described by Eqs. 1, 4–6, and 8 using a Hill coefficient of 1, which suggests that QX314 blockade occurred in an all-or-none fashion.

Ryanodine modifies the SR Ca^{2+} release channel by inducing the formation of an open, subconducting channel state with about half the unitary Ca^{2+} (Rousseau et al., 1987) and K^+ (Fig. 7) conductances of the unmodified channel. Since ryanodine also decreases the rate constant of glucose efflux from SR vesicles (Meissner, 1986), it is conceivable that ryanodine modification results in a narrowing of the channel's conductance pathway. Ryanodine also profoundly affected the kinetics of QX314 blockade. Zero-voltage blocking and unblocking rates were decreased by factors of ~35 and 7.5, respectively. Further, the voltage dependence of the blocking rate increased by a factor of about two, whereas that of the unblocking rate was only moderately affected, after ryanodine modification. The more pronounced changes of ryanodine on the blocking rate led to a decreased affinity but increased voltage dependence of the overall blocking reaction for the ryanodine-modified channel. Additional studies will be required to more fully characterize the block by QX314 of the ryanodine-modified and -unmodified channels.

Block of Ryanodine-modified Channel by Tetracaine and Procaine

A blocking action of tetracaine on the Ca^{2+}-gated, ryanodine-unmodified channel could not be demonstrated. One likely reason was that binding of tetracaine to its higher affinity regulatory sites reduced channel P_o to very low values before single channel blockade could have been discerned at the low frequency resolution of the bilayer system. A fast procaine block of the ryanodine-unmodified channel occasionally took place; however, because of its infrequent occurrence it was not further studied.

Ryanodine modification rendered the SR Ca^{2+} release channel less sensitive to inhibition by tetracaine and procaine. Under these conditions, a fast block of ryanodine-modified channel by the two local anesthetics could be consistently seen. In the ryanodine-modified channel, the presence of high (>1 mM) tetracaine concentrations on either or both sides of the bilayer caused asymmetric blocking,

lowering single channel conductance and channel P_o for positive holding potentials only. The channel conductance and open time were only minimally affected at negative potentials. Since this asymmetry resulted regardless of which side of the bilayer the drug was added to, it is clear that tetracaine had ready access to its blocking site from both sides of the bilayer. The voltage-dependent block by tetracaine could not be well fit to a simple blocking scheme as described by Eqs. 1 and 7 in Results. One possible reason was that tetracaine, because of its high lipid solubility, could effectively reach the blocking site via a route different from that of the conduction pathway, such as the SR membrane composed of phospholipid and transmembrane spanning segments of the SR Ca^{2+} release channel. Alternatively, it was possible that the modified channel underwent a conformational change at positive voltages, such that the channel could be physically blocked only at these voltages. On the other hand, fast *cis* procaine blockade of the ryanodine-modified channel could be well described by a simple blocking scheme. As summarized in Table I, zero-voltage blocking and unblocking rates of procaine were about four times faster than those of QX314. Another difference was that the unblocking rate of procaine appeared to be nearly voltage independent, suggesting that the main voltage-dependent step of the blocking reaction resided in the blocking step. A similar pattern, with k_b being voltage dependent but k_{-b} being largely voltage independent, has been reported for Na^+ block of the Ca^{2+}-activated K^+ channel (Yellen, 1984), bis Q10 block of the SR K^+ channel (Miller, 1982), and Cs^+ block of the ATP-sensitive K^+ channel (Quayle, Standen, and Stanfield, 1988). These results have been explained in terms of a diffusional limitation of ion flow into the mouth of the channel's pore (Yellen, 1984) and by the formation of a tight ion complex between the cationic blocker and a carboxylic group which may have given rise to a voltage-independent unblocking rate (Moczydlowski, 1986). It would appear that neither explanation readily applies to the SR Ca^{2+} release channel since procaine binding affinities were low and the drug concentrations used were fairly high. One alternative explanation for a largely voltage-independent unblocking rate would be that binding of procaine resulted in the formation of an uncharged molecule.

Inhibition of SR Ca^{2+} Release by Local Anesthetics

In earlier studies (see Introduction) it has been proposed that a primary target of tetracaine and procaine in E-C coupling is a Ca^{2+} release pathway in the SR. In two recent studies it was suggested that tetracaine, by inhibiting SR Ca^{2+} release, affects the kinetics of one of the components (Q_γ or hump) of charge movement in skeletal muscle (Szucs et al., 1991; Pizarro et al., 1991). Although concentrations of tetracaine as low as 20 and 25 μM were effective, Szucs et al. (1991) also obtained indirect evidence that such low concentrations of tetracaine were not sufficient to remove all of the tetracaine-sensitive charge. The results of this study provide the first more direct insights into the mechanism by which local anesthetics may affect E-C coupling in skeletal muscle. Our results suggest that tetracaine and procaine reversibly inhibit SR Ca^{2+} release under different activating conditions by inducing protein conformational changes that result in a closing of the SR Ca^{2+} release channel. The presence of cooperatively interacting drug binding sites leads us to suggest that there may be four regulatory drug binding sites per tetrameric Ca^{2+} release channel complex (Fig.

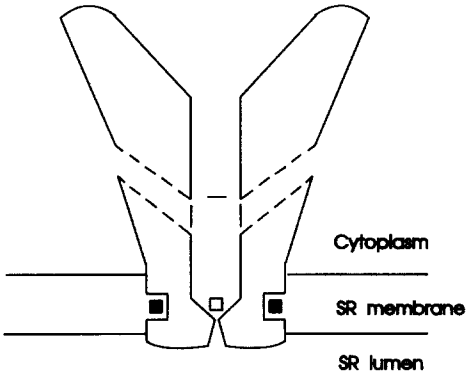

FIGURE 11. Model of SR Ca^{2+} release channel depicting proposed location of regulatory and blocking sites for local anesthetics. Shown is a cross-section of the ion conductance pathway of the tetrameric SR Ca^{2+} release channel composed of a central channel that may (Wagenknecht et al., 1989) or may not be connected to four radial channels located in the cytoplasmic portion of the four subunits (only two are shown) of the channel. The model also indicates (*a*) the presence of four (only two are shown) high-affinity, regulatory binding sites for local anesthetics (■) in or near the SR membrane, and (*b*) a low-affinity blocking site for local anesthetics (□) which is proposed to be located in the central, transmembrane spanning segment of the ion conductance pathway.

11). Cooperative interactions within the skeletal muscle SR Ca^{2+} release channel complex have been previously suggested (*a*) to explain the appearance of up to four equivalent conductance states (Smith et al., 1988; Liu et al., 1989), (*b*) in studies using the specific channel ligand ryanodine (Lai, Misra, Xu, Smith, and Meissner, 1989), and (*c*) by studying the interaction of the channel with its three regulatory ligands Ca^{2+}, Mg^{2+}, and ATP (Meissner et al., 1986). In addition to an interaction with regulatory sites, local anesthetics may also inhibit SR Ca^{2+} release by binding to a blocking site present within a centrally located conductance pathway of the channel (Fig. 11). We suggest that, because of a lower affinity of the blocking site, the two local anesthetics tetracaine and procaine primarily inhibit the release of Ca^{2+} ions from SR in muscle by an allosteric rather than a direct blocking mechanism.

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