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The Interaction of Local Anesthetics with the Ryanodine Receptor of the Sarcoplasmic Reticulum

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Summary. The effects of various local anesthetics (LAs) on the skeletal muscle ryanodine receptor were tested. The LAs were divided into three categories according to their effects on the binding of ryanodine to the junctional sarcoplasmic reticulum membranes. Ryanodine binding was assayed in the presence of 0.2 M NaCl and 10 μM $CaCl_2$. Tetracaine and dibucaine inhibit the binding with half-maximal inhibition (CI_{50}) of 0.12 and 0.25 mM, respectively, while inhibition by benzocaine and procaine occurs with CI_{50} of about 10-fold higher. Lidocaine, its analogue QX-314, and prilocaine, on the other hand, stimulate the binding up to fourfold with half-maximal stimulation occurring with about 2 mM of the drugs. Lidocaine increases both the receptor affinity for ryanodine by about fivefold and the rate of ryanodine association with its binding site by about 10-fold.

Tetracaine interacts with the ryanodine receptor in a noncompetitive fashion with respect to ryanodine but it competes with lidocaine for its binding site, suggesting the existence of a single site for the inhibitory and stimulatory LA.

The LAs also interact with the purified ryanodine receptor and produce effects similar to those with the membrane-bound receptor.

Tetracaine and dibucaine inhibit binding of the photoreactive ATP analogue; [α-^{32}P]benzoyl-benzoyl ATP (BzATP) to the ATP regulatory site of the ryanodine receptor, and high concentrations of ATP decrease the degree of ryanodine binding inhibition by tetracaine, indicating the relationship between the receptor conformations stabilized by ATP and LAs.

Based on a structure-activity relationship, a model for the LA site of interaction in the ryanodine receptor is suggested.

Key Words local anesthetics · ryanodine receptor · sarcoplasmic reticulum · Ca^{2+} release channel

Introduction

Contraction in skeletal muscle is initiated by a depolarization of the transverse tubular membrane which, in turn, signals the release of Ca^{2+} from the sarcoplasmic reticulum (SR) [12, 40]. A key protein involved in the excitation-contraction coupling is the ryanodine receptor—a protein which binds the toxic alkaloid ryanodine with nanomolar affinity [28].

The purified protein has been found to consist of high molecular weight polypeptides (~450 kD) which are assembled into a tetrameric complex of apparent sedimentation coefficient 30S [8, 20, 27]. Recently, the cDNA of the ryanodine receptor from skeletal muscle has been cloned and sequenced, and a molecular mass of 565 kD was determined [42, 48]. When incorporated into planar lipid bilayers, the purified protein exhibits a calcium conductance with pharmacological properties of the native SR Ca^{2+} release channel [19, 27]. Structural analysis of the purified receptor suggests that it corresponds to the junctional feet structures which connect the SR junctional face membranes to the transverse tubule [20, 27].

Several investigations have been undertaken on the effects of local anesthetics (LAs) on skeletal and cardiac muscle sarcoplasmic reticulum activities [1–3, 9, 13, 14, 16, 21, 22, 31, 32, 34, 37, 41, 46]. However, it has been observed that LAs drugs exert complex effects on SR. Several local anesthetics were found to inhibit Ca^{2+} release induced by a variety of methods [2, 16, 31]. On the other hand, some LAs are known to cause a contraction by themselves [3, 16, 21, 34, 46]. However, whereas dibucaine enhances the Ca^{2+}-induced Ca^{2+} release [46], prilocaine does not facilitate this mechanism, although it increases the release of Ca^{2+} from the SR [34]. Recently [37], we have shown that preincubation of SR with LAs in the presence of ATP stimulates Ca^{2+} efflux from the SR vesicles. Thus, the complex effects of LAs on the behavior of the SR suggest that these agents may either have multiple sites of action and/or their structure and the conditions under which they are manipulated control their effects. Their mode of action, however, is still to be determined.

In this study we performed a detailed analysis of the interaction of several LAs with membrane-

bound and purified ryanodine receptor. We show that the LAs interact with the ryanodine receptor and either stimulate or inhibit the binding of ryanodine to its receptor. A model for the structure-activity relationship is suggested.

Materials and Methods

Materials

ATP, EGTA,[1] Tris, Tricine, MOPS, procaine, tetracaine, benzocaine, prilocaine, chlorpromazine, dibucaine, and lidocaine were obtained from Sigma. [^{3}H]ryanodine (60 Ci/mmol) was purchased from New England Nuclear. Unlabeled ryanodine was obtained from Calbiochem.

Benzocaine, lidocaine were dissolved in methanol. The last two were then diluted 1 : 4 with H_2O while stirring and the pH was adjusted to about 7.0. The final methanol concentration in control and LA-containing samples never exceeded 2%.

Membrane Preparations

Junctional SR membranes were prepared from rabbit fast-twitch skeletal muscle as described by Saito et al. [35]. In most of the experiments the fraction R_4 was used. The membranes were suspended to a final concentration of about 25 mg protein/ml in a buffer containing 0.25 M sucrose, 10 mM tricine, pH 8.0 and 1 mM histidine and stored at −70 °C. Protein concentration was determined by the method of Lowry et al. [29].

Purification of the Ryanodine Receptor

Ryanodine receptor was purified by the spermine-agarose method [38]. The purified protein (30–70 μg/ml) was assayed for [^{3}H]ryanodine binding (in 0.1 ml) as described below for the membranes, except that soybean lecithin (5 mg/ml) was present in the assay medium. After 2 hr at 30 °C, the bound ryanodine was assayed by polyethylene glycol 600 (PEG) precipitation in the presence of carrier protein (1.4 mg/ml BSA), followed by filtration through Whatman GF/B filters and 3 × 4 ml washes with 10% PEG solution [38].

[^{3}H]Ryanodine Binding

Unless otherwise specified, junctional SR membranes (final concentration of 0.5 mg/ml) were incubated for 2 hr at 37 °C with 20 nM [^{3}H]ryanodine (Spec. act. = 30 Ci/nmol), in a standard binding solution containing either 0.2, 0.5 or 1 M NaCl, 20 mM MOPS, pH 7.4 and 10 μM $CaCl_2$. Unbound ryanodine was separated from protein-bound ryanodine by filtration of protein aliquots (50 μg) through Whatman GF/C filters, followed by washing three times with 5 ml of ice-cold buffer containing 0.2 M NaCl, 10 mM MOPS, pH 7.4 and 50 μM $CaCl_2$. The filters were dried, and the retained radioactivity was determined by standard liquid scintillation counting techniques. Specific binding of [^{3}H]ryanodine is defined as the difference between the binding in the absence and presence of 20 μM unlabeled ryanodine.

Results

The effects of various concentrations of several local anesthetics (LAs) (their structures are shown in Fig. 1) on ryanodine binding by junctional SR membranes are illustrated in Fig. 2. These compounds are divided into three categories according to their effects on ryanodine binding to the membrane-bound ryanodine receptor. Tetracaine and dibucaine (Fig. 2*A*) inhibit ryanodine binding with half-maximal inhibition (CI_{50}) occurring at about 0.12 and 0.25 mM for tetracaine and dibucaine, respectively. Dibucaine alters ryanodine binding in a biphasic manner—stimulation at low concentrations (>100 μM) and inhibition at higher concentrations (Fig. 2*A*). Chlorpromazine, whose structure resembles that of a typical LA but missing the carbonyl group, stimulates ryanodine binding between 25 to 200 μM and inhibits the binding at higher concentrations (Fig. 2*A*). Chlorpromazine is considered to be a calmodulin antagonist but has been shown to act as a LA [25]. Procaine and benzocaine are much less potent; 50% inhibition is obtained with 2 and 6 mM of procaine and benzocaine, respectively (Fig. 2*B*). Lidocaine, its analogue QX-314, and prilocaine, on the other hand, stimulate the binding of ryanodine up to threefold (Fig. 2*C*).

Table 1 summarizes the effects of the various LAs and other compounds, such as caffeine and chlorpromazine, on ryanodine binding by presenting the concentration of the drug required for 50% inhibition (CI_{50}) or stimulation (CS_{50}) of ryanodine binding, as well as the pKa values of the various drugs. The structure-activity relationship of these drugs is discussed below.

The inhibitory and stimulatory effects of LAs on ryanodine binding are strongly dependent on the assay conditions. Figure 3 shows the effect of NaCl concentration on ryanodine binding with and without tetracaine. As shown previously [20, 30], high NaCl concentrations stimulate ryanodine binding. The degree of inhibition of ryanodine binding by tetracaine is dependent on the NaCl concentration present in the ryanodine binding medium, decreasing as the NaCl concentration increased (Fig. 3*B*). The influence of NaCl concentration on the effects of LAs on ryanodine binding is summarized in Table 2. The data indicate that the effect of dibucaine on ryanodine

[1] Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N′N′-tetraacetic acid; Tricine, N-[2-hydroxy-1, 1-bis (hydroxymethyl)-ethyl]-glycine, MOPS, 3-(N-morpholino) propanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum; PEG, polyethylene glycol 600; LAs, local anesthetics.

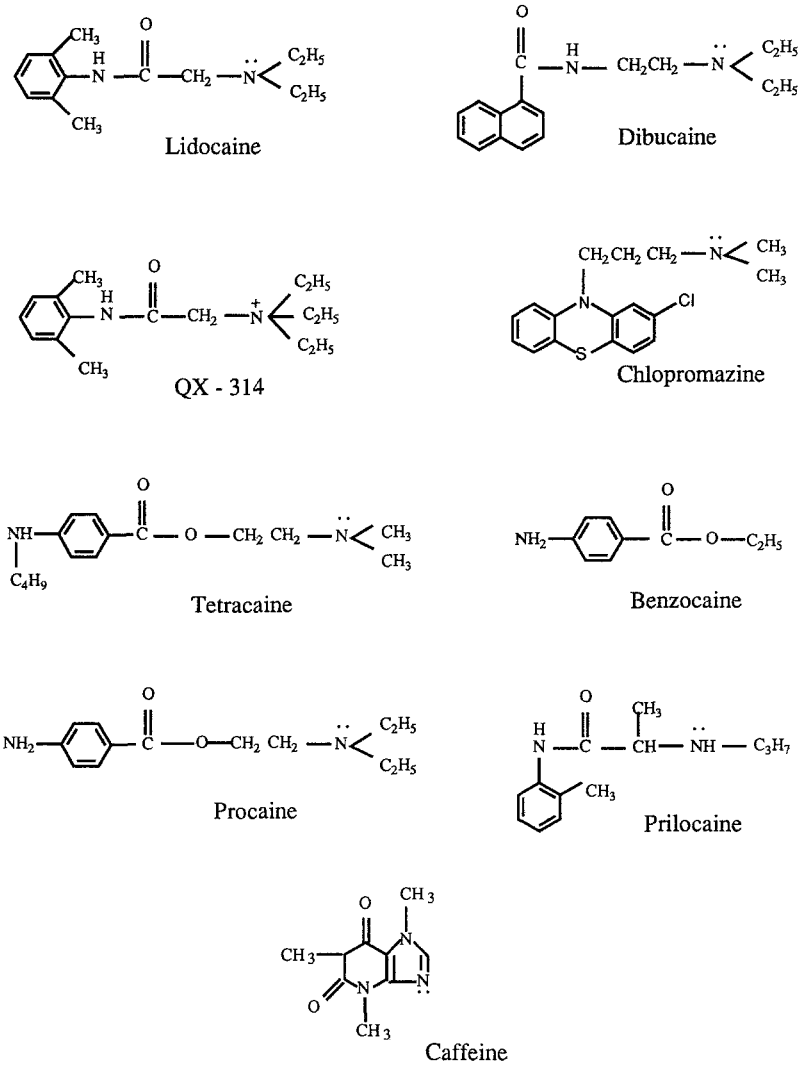

Fig. 1. Structures of caffeine and local anesthetics discussed in this paper.

binding is also dependent on ionic strength. Dibucaine, at the concentrations used, produces inhibition at 0.2 M NaCl and stimulation at 1.0 M NaCl. The stimulation of ryanodine binding by lidocaine is decreased with increasing NaCl concentration, from stimulation of about 3.6-fold at 0.2 M NaCl to 1.4-fold in the presence of 1.0 M NaCl. This effect of NaCl is expected, since, in the presence of 1.0 M NaCl, ryanodine binding is close to its maximal level. These results suggest that the LA site of interaction is either influenced directly by ionic strength or indirectly due to NaCl stabilization of a protein conformation with modified LA binding site.

The effect of pH on ryanodine binding and on the inhibition of ryanodine binding by tetracaine is shown in Fig. 4. As has been shown previously [30], the binding of ryanodine is pH dependent, increasing at alkaline pH up to 8.5. Tetracaine inhibits the binding of ryanodine at all the pH values tested. The degree of inhibition by tetracaine is affected only slightly by the assay pH, decreasing from about 98 to 77% by a shift in pH from 6.1 to 8.5.

Table 3 shows the effect of ATP on the degree of inhibition of ryanodine binding by tetracaine and dibucaine. As with NaCl, ATP decreases the degree of inhibition of ryanodine binding by both drugs, while on the other hand, ATP does not interfere with stimulation by lidocaine.

Figure 5 shows the binding of ryanodine as a function of its concentration in the absence and presence of tetracaine. Double reciprocal plots of the data (Fig. 5*B*) are consistent with a noncompetitive inhibition by tetracaine for the ryanodine binding sites. Similar results are obtained with dibucaine (*data not shown*). Thus, the results show that tetracaine interacts with the ryanodine receptor in a noncompetitive fashion with respect to ryanodine.

Figure 6 shows an experiment similar to that in

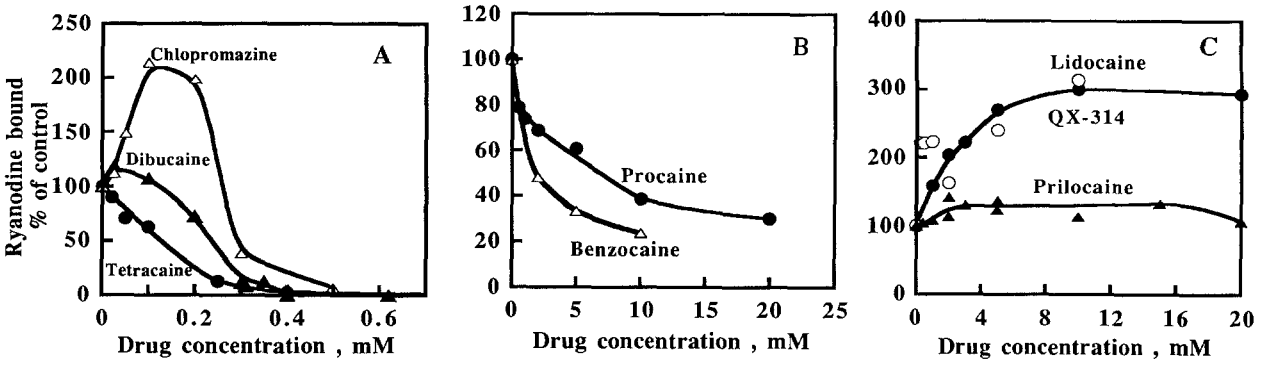

Fig. 2. Effect of different local anesthetics on ryanodine binding. Junctional SR membranes (0.5 mg/ml) were assayed for ryanodine binding in 0.2 M NaCl in the absence and presence of the indicated LAs as described in Materials and Methods. Control Activity (100) = between 3.0 to 3.8 pmol ryanodine bound/mg protein.

Table 1. Effect of different drugs on ryanodine binding by SR membranes

Compound	pKa	CI_{50} mM	CS_{50} mM
Dibucaine	8.5	0.25	
Chlorpromazine		0.27	0.08
Tetracaine	8.5	0.12	
Benzocaine	2.6	2.0	
Procaine	8.9	6.0	
Prilocaine	7.8		2.0
Lidocaine	7.9		2.0
QX-314	UP*		2.0
Caffeine	0.8		2.0

[^{3}H]Ryanodine binding was assayed in 0.2 M NaCl in the absence and presence of different concentrations of the indicated LAs, as described in Fig. 2. Control activity for [^{3}H]ryanodine binding (100%) was 2.5 pmol/mg protein. The drug concentration which produced 50% inhibition (CI_{50}) or stimulation (CS_{50}) of ryanodine binding is shown. The pKa values of the various drugs are also given.* Undefined parameter.

Fig. 5, except that the effect of lidocaine is tested. Scatchard plot analysis of ryanodine binding in the absence and presence of 6 mM lidocaine indicates that lidocaine increases the apparent binding affinity (K_D) about 5.6-fold, from 79 ± 8 nM (n = 3) to 14 ± 4 nM (n = 2). In contrast, similar B_{max} values are obtained with 0.2 M NaCl ± 6 mM lidocaine (B_{max} = 16 ± 2 pmol/mg protein (n = 3). Thus, the results suggest that the increase in ryanodine binding produced by lidocaine (at low NaCl concentration) is attributable to increased receptor affinity for the ligand.

The effect of lidocaine on ryanodine association with and dissociation from its binding site is shown in Fig. 7. Lidocaine increases ryanodine binding by three to fourfold during the period of time tested (5–120 min Fig. 7*A*, inset). Kinetic analysis of the data (Fig. 7*A*) indicates that lidocaine increases the observed association rate constant (K_{obs}). Ryanodine associates with its binding site with K_{obs} of 0.027 and 0.07 min^{-1} in the absence and presence of lidocaine, respectively. The calculated pseudo-first-order association rate constants (K_1) obtained in the absence and presence of lidocaine are: 2 × 10^4 and 26 × 10^4 M^{-1} min^{-1}, respectively.

Dissociation of bound ryanodine at equilibrium is initiated by 80-fold dilution (Fig. 7*B*). A similar monophasic dissociation is obtained in the presence and absence of 6 mM lidocaine (K_{-1} = 0.0012 min^{-1}).

The K_D calculated from association and dissociation constants are: 44 and 4.8 nM in the absence and presence of lidocaine, respectively, which are about twofold lower than the K_D calculated from saturation experiments (Fig. 6).

The relationship between lidocaine (stimulatory LA) and tetracaine (inhibitory LA) binding sites is demonstrated in Fig. 8. The data are consistent with a competition of tetracaine for the lidocaine binding site. On the other hand, caffeine stimulation of ryanodine binding is inhibited by tetracaine in a non-competitive fashion; increasing caffeine concentration by 10-fold decreases the inhibition by tetracaine only from 88 to 79% (*data not shown*). These observations may suggest the existence of a single site for the inhibitory and stimulatory LA which is distinct from the caffeine binding site.

The effects of LAs on the ATP-binding site were tested using the photoreactive derivative of ATP, (Benzoyl)benzoyl ATP (BzATP) [*see* 47]. Irradiation of junctional SR membranes with [α-^{32}P]BzATP results in covalent binding of the label to several SR proteins (Fig. 9). The labeling pattern obtained, subsequent to SDS-PAGE, of junctional SR membranes that had been photolabeled with [α-^{32}P]BzATP, shows photoincorporation of the label

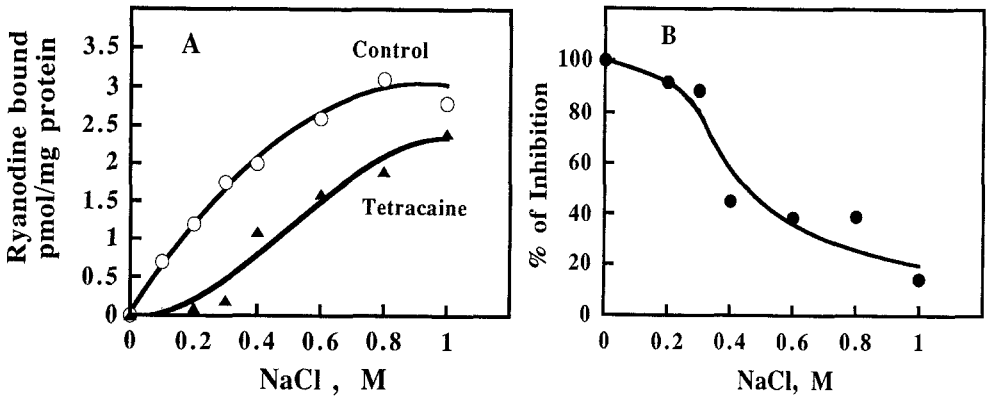

Fig. 3. Effect of NaCl concentration on ryanodine binding and on the degree of inhibition of ryanodine binding by tetracaine. Junctional SR membranes (0.5 mg/ml) were assayed for ryanodine binding as described under Materials and Methods, except that the NaCl concentration was varied as indicated, in the absence (○) and presence of 0.25 mM tetracaine (▲). In *B*, % of inhibition by tetracaine is presented as a function of NaCl concentration.

Table 2. Effect of NaCl on the degree of inhibition or stimulation of ryanodine binding by LAs

Local Anesthetic	Concentration mM	[^{3}H] Ryanodine bound pmol/mg protein	
		0.2 M NaCl	1.0 M NaCl
None		3.35	11.3
Tetracaine	0.3	0.35	6.5
Tetracaine	0.6	0.22	3.7
Dibucaine	0.4	4.68	13.5
Dibucaine	0.8	0.29	17.9
Procaine	6.0	1.63	6.2
Procaine	12.0	0.90	4.9
Lidocaine	1.0	5.00	12.5
Lidocaine	3.0	8.46	13.7
Lidocaine	6.0	12.16	15.8

Ryanodine binding was assayed as in Fig. 2 in the presence of 0.2 or 1.0 M NaCl and in the absence or presence of the indicated concentration of the LA.

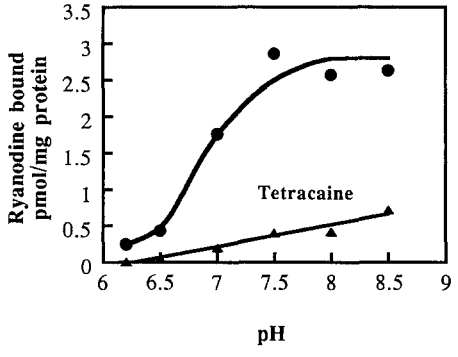

Fig. 4. pH-dependence of ryanodine binding in the absence and presence of tetracaine. SR membranes (0.5 mg/ml) were assayed for ryanodine binding in the absence (●) or presence (▲) of 0.3 mM tetracaine as described in Materials and Methods, except that the pH was varied as indicated. The buffers used were; 20 mM MES for the pH values 6.1, 6.4 and 7.0; 20 mM MOPS for pH 7.5 and 8.0 and 20 mM Tricine for pH 8.5.

into several proteins including: the 200 kD protein (myosin), the 110 kD (Ca^{2+}-ATPase), the 97 kD (phosphorylase-b), the 48 kD protein and the 450 kD protein (ryanodine receptor). Tetracaine, dibucaine and procaine prevent the labeling of the 450 kD protein band, but not of other proteins. A more quantitative analysis was carried out by densitometric scanning of the autoradiogram to determine the peak area of the 450 kD band, and the results are also presented in Fig. 9.

The effects of different LAs and other reagents on ryanodine binding to the soluble and to the purified ryanodine receptor are shown in Table 4. As with the membrane-bound receptor, tetracaine and procaine inhibit and lidocaine stimulates the binding to the solubilized membranes and to the purified receptor in the same range of concentrations. Thus, the experiments in Table 4 indicate that the drugs tested interact directly with the ryanodine receptor

and that their binding site is preserved in the purified receptor.

Discussion

In this study, the effects of local anesthetics on ryanodine binding to the membrane-bound and purified ryanodine receptor are presented. We found that dibucaine, tetracaine, procaine and benzocaine inhibit ryanodine binding to both membrane-bound and purified ryanodine receptor, while lidocaine and QX-314 stimulate ryanodine binding up to fourfold. The potency of the LAs is strongly dependent on the assay conditions. The presence of high NaCl concentration or ATP, which both stimulate ryanodine binding [20, 30], diminish inhibition by LAs. It is possible that receptor conformation stabilized by high ionic strength or ATP is much less sensitive to LAs. The relationship between the receptor conformational states stabilized by ATP and LAs is also reflected by the inhibitory effect of LAs on the labeling of ryanodine receptor by the photoreactive analogue of ATP, Bz-ATP (Fig. 9). This suggests that the protein conformation stabilized by LAs may be less recognized by ATP.

The present study indicates a specific interaction of the LAs with the skeletal muscle ryanodine receptor. The interaction of LAs with the purified ryanodine receptor suggests that the receptor possesses an intrinsic binding site or sites for LAs.

The mechanisms by which LAs exert their effects on nerves and muscles have been discussed extensively [4, 7, 16, 33]. Several sites of interaction have been suggested, including the hydrocarbon core of the lipid bilayers [10], a surface interaction with Ca^{2+} binding sites on the phospholipids [25], or a direct interaction with a putative receptor [18]. Specific interactions of LAs with Na^+ channel [7, 36], acetylcholine receptor [17], Ca^{2+} channels [6, 23], and K^+ channels [23] have been suggested previously. The findings that LAs modify the activities of various channels may suggest the presence of a common site for LAs in the different ionic channels.

Several hypotheses concerning the nature of the binding sites and the mechanism of binding have been formulated for the interaction of LAs with lipids [39] or with specific receptors [4, 11, 18, 33]. It has been indicated that all effective LAs are characterized by a carbonyl group capable of forming hydrogen bounds. Thus, involvement of the carbonyl group in the interaction of LAs with their receptors in the nerve cells has been suggested [4, 11].

Table 3. Effect of ATP on the inhibition and stimulation of ryanodine binding by local anesthetics

Additions	[^{3}H]Ryanodine bound pmol/mg protein	
	−ATP	+ATP
None	3.3 (100)	14.7 (100)
Tetracaine (0.2 mM)	0.6 (18)	6.5 (45)
Tetracaine (0.4 mM)	0.27 (8)	4.6 (32)
Lidocaine, 3 mM	13 (390)	21.9 (150)
Lidocaine, 10 mM	12.2 (370	21.5 (147)

[^{3}H]Ryanodine binding was assayed as in Fig. 2 in the absence or presence of the indicated LAs and/or 2.5 mM ATP. The free Ca^{2+} concentration was 10 μM in the absence and presence of ATP. The numbers in parentheses indicate percent of control.

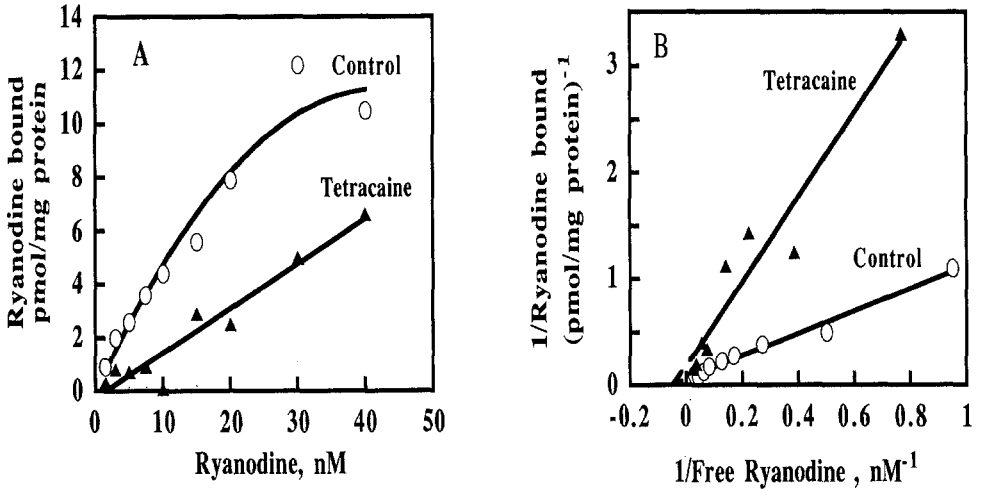

Fig. 5. Tetracaine inhibition of [^{3}H]ryanodine binding to SR membranes. [^{3}H]Ryanodine binding, in the absence (○) and presence of tetracaine (0.4 mM) (▲) was carried out as in Fig. 2, except that NaCl concentration was 0.5 M. The double-reciprocal plot of the data is presented in *B*. The K_m for [^{3}H]ryanodine is 20 nM. This is a representative experiment.

To obtain some clues about the nature of LA site of interaction and about the molecular features involved in the binding of LAs to their receptor, we carried out an analysis of a structure-activity relationship. This analysis reveals the structural characteristics involved in the binding/action of the LAs. It has been suggested [3, 4] that LA activity is a function of the relative concentration of the charged and uncharged forms of the tertiary amine group. The uncharged form of tetracaine or procaine is responsible for inducing Ca^{2+} release, whereas the charged form is responsible for inhibition of Ca^{2+} release. However, this suggestion has been questioned [32, 37]. Our results (Table 1) show that ryanodine binding is inhibited by either the protonated or neutral forms of LAs. Also, there is no apparent relationship between the effectiveness of LA inhibition of ryanodine binding and the pKa. Thus, dibucaine (pKa, 8.5) and tetracaine (pKa, 8.5) are much more active than procaine (pKa, 8.9) or lidocaine (pKa, 7.9) which stimulate ryanodine binding.

The observation that different LAs have inhibitory or stimulatory effects on ryanodine binding raises the question of whether the two modes of action are due to LA interaction at distinct sites; namely, is there a single site or multiple sites for LA action. The results in Fig. 8 support the involvement of a single site for LAs that could bind either the inhibitory or the stimulatory LA, but probably not bind caffeine. This suggests that the LA binding site is one of little structural specificity and, there-

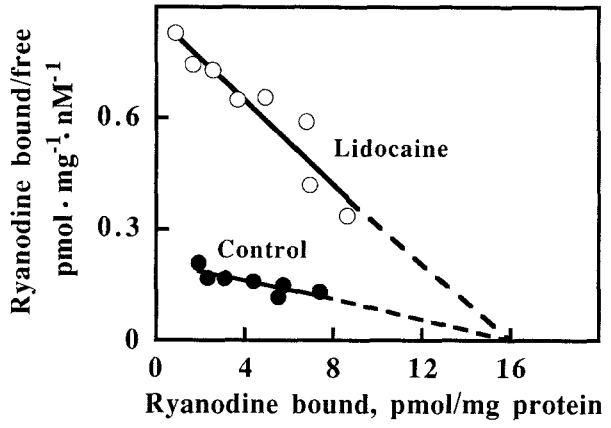

Fig. 6. The influence of lidocaine on the affinity of the ryanodine binding site. [^{3}H]Ryanodine was assayed as in Fig. 2 in the presence of 0.2 M NaCl, in the absence (●) and presence (○) of 6 mM lidocaine, except that [^{3}H]ryanodine concentration was varied and [^{3}H]ryanodine was diluted 1 : 1 with unlabeled ryanodine. The K_D values calculated from the Scatchard plots were: 75.6 and 18.4 nM in the absence and presence of lidocaine, respectively.

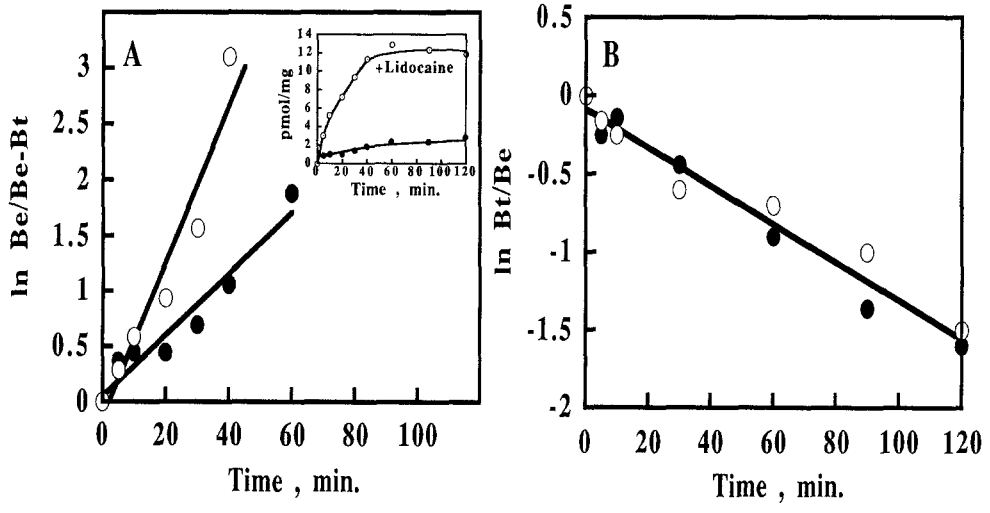

Fig. 7. Effect of lidocaine on equilibrium binding of [^{3}H]ryanodine and its dissociation kinetics. (*A*) SR membranes (0.5 mg/ml) were incubated with 20 nM ryanodine at 37 °C, as in Fig. 2, in the absence (●) and presence (○) of 6 mM lidocaine. After the indicated incubation time, aliquots were assayed for bound ryanodine (Bt). The maximal amount of ryanodine bound at the plateau (Be) was 3.05 and 12.9 pmol/mg protein, in the absence and presence of lidocaine, respectively. The time course for ryanodine binding in the absence and presence of lidocaine is shown in the inset. (*B*) SR membranes were incubated with 20 nM ryanodine as in *A*. After 2 hr, aliquots were assayed for bound ryanodine (Be = 3.05 pmol/mg protein). Dissociation of bound ryanodine was initiated by 80-fold dilution with the binding medium (without ryanodine) with (○) and without (●) 6 mM lidocaine, and determination of the residual ryanodine bound at the indicated time (Bt). The calculated K_{-1} was 0.0012 min^{-1} in the absence and presence of lidocaine. K_1 was calculated from the K_{obs}, as described previously [44], using the following equation: $K_{obs} = K_1[L][R]/Be$, where [L] = ryanodine concentration, [R] = ryanodine receptor concentration = B_{max} (16.5 pmol/mg protein). $K_1 = 2.5 \times 10^4$ and 26×10^4 M^{-1} min^{-1}, in the absence and presence of lidocaine, respectively. This is one of three similar experiments.

Table 4. Comparison of the effect of local anesthetics on ryanodine binding to the membrane-bound and the purified ryanodine receptor

Additions	Concentration mM	[^{3}H]Ryanodine bound % of control		
		Membrane-bound	Solubilized	Purified
None		100	100	100
Tetracaine	0.3	35	45	47
Tetracaine	0.6	17	23	22
Procaine	3.0	68	58	63
Procaine	7.0	46	43	40
Lidocaine	3.0	167	128	132
Lidocaine	6.0	210	131	162

The binding of [^{3}H]ryanodine (20 nM) to the SR membranes, or solubilized membranes (0.5 mg/ml) and to purified receptor (30–45 μg/ml) was determined as described in Materials and Methods, except that NaCl concentration was 0.5 M and the indicated compounds were added to the assay medium. Control activities (100%) were : 6.6, 9.7 and 150 pmol/mg protein for membrane-bound solubilized membranes and purified receptor, respectively. Results are the average of three experiments for the membrane-bound and purified receptor.

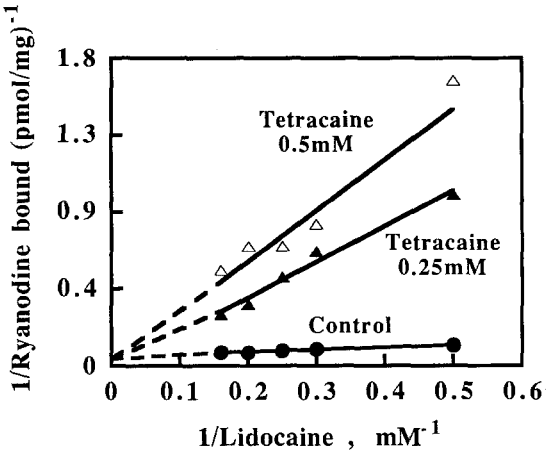

Fig. 8. Tetracaine inhibited the ryanodine binding stimulated by lidocaine. [^{3}H]Ryanodine binding was assayed as in Fig. 2 in the absence and presence of the indicated lidocaine concentrations and without and with 0.25 or 0.5 mM tetracaine. This is one of three similar experiments.

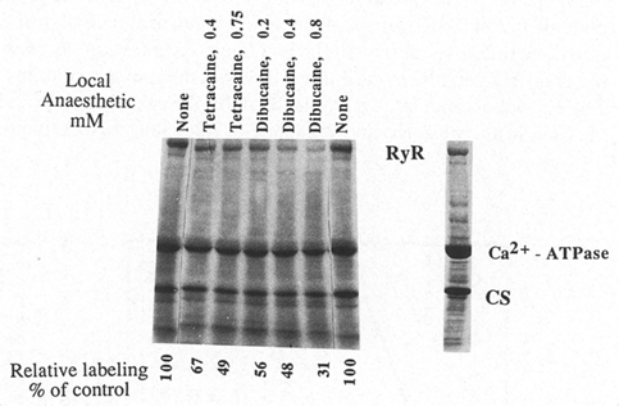

Fig. 9. Effect of local anesthetics on photoaffinity labeling of SR proteins by [α-^{32}P]BzATP. [α-^{32}P]BzATP was synthesized and purified as described by Williams and Coleman [45]. SR membranes (1 mg/ml) were irradiated in the presence of 1 μM [α-^{32}P]BzATP (4 × 10^6 cpm/nmol) in 75 μl of 25 mM MOPS, pH 7.4, 0.4 M NaCl and 0.5 mM EDTA, using a 15W UV lamp for 3 min at a distance of 5 cm. The irradiated membranes were denatured for 3 min at 95 °C in 125 mM Tris-HCl, pH 6.8; 10% glycerol; 2% SDS and 2% β-mercaptoethanol. The samples were analyzed by SDS-PAGE according to Laemmli [26] using 4–13% linear polyacrylamide gradient gel and 3.5% stacking gel. Autoradiography of the dried gels was carried out using Kodak X-OMAT film. Quantitative analysis of the labeled protein bands was determined by densitometric scanning of the autoradiogram, using a Molecular Dynamics personal scanning densitometer (shown in the bottom of the autoradiogram). The autoradiogram is on the left and the Coomassie stain of one lane of the gel is on the right. RyR, ryanodine receptor; CS, calsequestrine and Ca^{2+}-ATPase, (Ca^{2+} + Mg^{2+}) ATPase. This is one representative experiment of three.

fore, the interaction of LAs with this site involves specific groups rather than general structure. Indeed, examination of the structures of the LAs (Fig. 1) indicates that their effectiveness is not related to the nature of the aromatic group or to the nature of the alkyl substitutions (dimethyl or diethyl) in the terminal amine group. Also, the hydrophilic bond (ester or amide) in the alkyl chain does not appear to play an important role in the LAs effectiveness. However, the structure-activity relationship shows inhibition of ryanodine binding by tetracaine and

Table 5. Summary of the effects of LAs on ryanodine binding and on Ca^{2+} release

Compound	Ryanodine binding	Ca^{2+} release or muscle contraction	References
Tetracaine	Inhibition	Inhibition	3[c],4[c],22[a],32[b],43[a]
		Stimulation	22[a],32[b]
Dibucaine	Stimulation/ Inhibition	Inhibition	22[a],46[b]
		Stimulation	3[c],46[b]
Procaine	Inhibition	Inhibition	3[c],4[c],15[b],22[a],24[a],32[a],43[a]
		Stimulation	32[b]
Benzocaine	Inhibition	Inhibition	4[c],43[a]
Prilocaine	Stimulation	Stimulation	34[b]
		Inhibition	43[a]
Chlorpromazine	Stimulation/ Inhibition	Stimulation	5[a]
Lidocaine	Stimulation	Stimulation	3[c],4[c]
		Inhibition	43[a]
QX-314	Stimulation	Inhibition[d]	43[a]

The references are for Ca^{2+} release and/or muscle contraction. The results for ryanodine binding are from this paper
[a] in isolated SR membranes
[b] in skinned muscle fibers
[c] in muscle fibers
[d] by high concentrations (5 mM) and with preincubation (3 hr).

dibucaine but not by QX-314, prilocaine or lidocaine, suggesting that the distance between the dipole in the carbonyl group and the nitrogen (cation) is essential for LA inhibitory activity (Figs. 1 and 2, Table 1). Lidocaine, prilocaine and QX-314, which stimulate ryanodine binding, show a smaller distance between the two groups compared with tetracaine or dibucaine. This suggestion is also supported by the stimulatory effect of chlorpromazine (<100 μM) on ryanodine binding. The structure of chlorpromazine resembles that of a typical LA (aromatic ring connected to a tertiary amine group by a short alkyl chain), but it lacks the carbonyl group present in all the other LAs tested (Fig. 1). Thus, this analysis of the results with respect to structure-activity relationship allowed us to propose a schematic LA binding site model which illustrates the regions of LA involved in binding.

Our proposed model for the nature of the LAs binding site and for their inhibitory and stimulatory interactions is presented in Fig. 10. We suggest that LAs bind to the ryanodine receptor, presumably via favorable interactions between their carbonyl oxygen and the positive charge on the tertiary amine located at the proper distance, and polar groups of the receptor (*site A* and *site B* in Fig. 10). Upon proper positioning of the binding groups, the LA interacts with its receptor and induces conformational changes which lead to inhibition of ryanodine

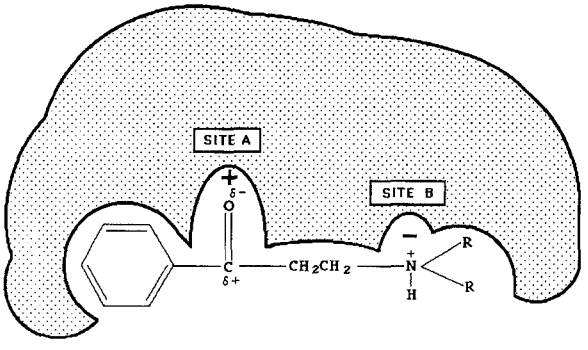

Fig. 10. A schematic model for the local anesthetics site of interaction in the ryanodine receptor. The model represents the interaction of tetracaine with the negative site (*Site B*) by the free pair of electrons of the nitrogen group and with the positive site (*Site A*) by the carbonyl oxygen. The model postulates the existence of a "protein-pocket" to which the hydrophobic part of the LA molecule binds.

binding. Interaction with only site B would lead to stimulation of ryanodine binding as produced by lidocaine, QX-314, prilocaine and chlorpromazine.

Bianchi [4] discussed a similar model in which he suggested the presence of positive and negative receptor sites, where the ability of the LAs to block caffeine-induced contracture or to produce contraction by themselves is dependent on the position of the LAs in these binding sites. He suggested also

that caffeine stimulates Ca^{2+} release by interacting with only one of the receptor electronegative group.

Various studies indicate that LAs affect the movement of Ca^{2+} across the SR membranes [1–3, 9, 13, 14, 16, 21, 22, 31, 32, 34, 37, 41, 46]. It has been shown that tetracaine and procaine are able to block, induce or potentiate Ca^{2+} release from SR, depending on the preparation and experimental conditions [22, 32, 43]. Lidocaine, in contrast, is found to release Ca^{2+} and to produce tonic muscle contraction [3, 4]. Thus, it is possible that the blocking or stimulating effects of LAs on Ca^{2+} release from SR are related to their interaction with the ryanodine receptor/Ca^{2+} release channel, which also results in inhibition or stimulation of ryanodine binding, respectively (Table 5).

In conclusion, according to Binachi [4] and our (Fig. 10) models, the dual effects of LAs can be explained on the basis of position of bonding groups on the LA and ryanodine receptor, which would be dependent not only on LA structure but also on the conditions and the LA concentrations used. Inhibition or stimulation of ryanodine binding might be reflected in inhibition or stimulation of Ca^{2+} release, respectively.

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References

1. Almers, W., Best, P.M. 1976. Effects of tetracaine on displacement currents and contraction in frog skeletal muscle. *J. Physiol.* **262:**583–611
2. Antoniu, B., Kim, D.H., Morii, M., Ikemoto, N. 1985. Inhibitors of Ca^{2+} release from the isolated sarcoplasmic reticulum. I. Ca^{2+} channel blockers. *Biochim. Biophys. Acta* **816:**9–17
3. Bianchi, C.P. 1968. Pharmacological actions on excitation-contraction coupling in striated muscle. *Fed. Proc.* **27:** 126–131
4. Bianchi, C.P., Bolton, P.C. 1967. Action of local anaesthetics on coupling systems in muscle. *J. Pharmacol. Exp. Ther.* **157:**388–405
5. Bindoli, A., Fleischer, S., 1983. Induced CA^{2+} release in skeletal muscle sarcoplasmic reticulum by sulfhydryl reagents and chlorpromazine. *Arch. Biochem. Biophys.* **221:**458–466
6. Bolger, G.T., Marcus, K.A., Daly, J.W., Skolnick, P. 1987. Local anaesthetics differentiate dihydropyridine calcium antagonist binding site in rat brain and cardiac membranes. *J. Pharmacol. Exp. Ther.* **240:**922–930
7. Butterworth, J.F., Strichartz, G.R. 1990. Molecular mechanism of local anesthesia. *Anesthesiology* **72:**711–734
8. Campbell, K.P., Knudson, C.M., Imagawa, T., Leung, A.T., Sutko, J.L., Kahl, S.D., Raab, C.R., Madison, L. 1987. Identification and characterization of the high affinity [^{3}H]ryanodine receptor of junctional sarcoplasmic reticulum Ca^{2+} release channel. *J. Biol. Chem.* **262:**6460–6463
9. Carvalho, A.P. 1968. Calcium binding properties of sarcoplasmic reticulum as influenced by ATP, quinidine and local anaesthetics. *J. Gen. Physiol.* **52:**622–644
10. De Boland, A.R., Jilka, R.L., Martonosi, A.N. 1975. Passive Ca^{2+} permeability of phospholipid vesicles and sarcoplasmic reticulum membranes. *J. Biol. Chem.* **250:**7501–7510
11. Elliott, J.R., Haydon, D.A., Hendry, B.M. 1984. Anaesthetic action of esters and ketones: evidence for an interaction with the sodium channel protein in squid axons. *J. Physiol.* **354:**407–418
12. Endo, M. 1977. Ca^{2+} release from sarcoplasmic reticulum. *Physiol. Rev.* **57:**71–108
13. Feinstein, M.B., Parimre, M. 1969. Pharmacological action of local anaesthetics on excitation-contraction coupling in striated and smooth muscle. *Fed. Proc., Fall Meeting of the Am. Soc. for Pharm. and Exp. Therap.* **28:**5
14. Fernandez-Belda, F., Soler, F., Gomez-Fernandez, J.C. 1989. Quinacrine inhibits the calcium-induced calcium release in heavy sarcoplasmic reticulum. *Biochim. Biophys. Acta* **485:**227–285
15. Ford, L.E., Podolasky, R.J., 1972. Calcium uptake and force development by skinned muscle fibers in EGTA buffered solutions. *J. Physiol.* **223:**1–19
16. Hebette, L., Messineo, F.C., Katz, A.M. 1982. The interaction of drugs with sarcoplasmic reticulum. *Annu. Rev. Pharmacol. Toxicol.* **72:**413–434
17. Heidman, T., Changeux, J.-P. 1979. Fast kinetic studies on the allosteric interactions between acetyl-choline receptor and local anesthetic binding site. *Eur. J. Biochem.* **94:**281–296
18. Hille, B. 1977. Local anaesthetics: hydrophilic and hydrophobic pathways for the drug-receptor reaction. *J. Gen. Physiol.* **69:**497–515
19. Hymel, L., Inui, M., Fleischer, S., Schindler, H. 1988. Purified ryanodine receptor of skeletal muscle sarcoplasmic reticulum forms Ca^{2+}-activated oligomeric Ca^{2+} channels in planar bilayers. *Proc. Natl. Acad. Sci. USA* **85:**441–445
20. Inui, M., Saito, A., Fleischer, S. 1987. Purification of the ryanodine receptor and identity with feet structures of junctional terminal cisternae of sarcoplasmic reticulum from fast skeletal muscle. *J. Biol. Chem.* **262:**1740–1747
21. Isaacson, A., Hinkes, M.J., Taylor, S.R. 1970. Contracture and twitch potentiation of fast and slow muscle of the rate at 20 and 37 °C. *Am. J. Physiol.* **218:**33–41
22. Johnson, P.N. Inesi, G. 1969. The effect of methylxanthines and local anaesthetics on fragmented sarcoplasmic reticulum. *J. Pharmacol. Exp. Therap.* **169:**308–314
23. Josephson, I.R. 1988. Lidocaine blocks Na, Ca and K currents of chick ventricular myocytes. *J. Mol. Cell Cardiol.* **20:**593–604
24. Kirino, Y., Shimizu, H. 1982. Ca^{2+}-induced Ca^{2+} release from fragmented sarcoplasmic reticulum: a comparison with skinned muscle fiber studies. *J. Biochem. (Tokyo)* **92:**1287–1296
25. Kwant, W.O., Seeman, P. 1969. The displacement of membrane calcium by a local anaesthetic (chlorpromazine). *Biochim. Biophys. Acta* **193:**338–349
26. Laemmli, U.K. 1970. Cleavage of structural proteins during

the assembly of the head of bacteriophage T_4. *Nature* **227:**680–685
27. Lai, F.A., Meissner, G. 1989. The muscle ryanodine receptor and its intrinsic Ca^{2+} channel activity. *Bioenerg. Biomembr.* **21:**227–245
28. Lai, F.A., Erickson, H.P., Rousseau, E., Liu, Q-Y., Meissner, G. 1988. Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature* **331:**315–319
29. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurements with folin phenol reagent. *J. Biol. Chem.* **193:**265–275
30. Michalak, M., Dupraz, P., Shoshan-Barmatz, V. 1988. Ryanodine binding to sarcoplasmic reticulum membranes; comparison between cardiac and skeletal muscle. *Biochim. Biophys. Acta* **939:**587–594
31. Morii, H., Tonomura, Y. 1983. The gating behaviour of a channel for Ca^{2+}-induced Ca^{2+} release in fragmented sarcoplasmic reticulum. *J. Biochem. (Tokyo)* **93:**1271–1285
32. Pike, G.K., Abramson, J.J., Salama, G. 1989. Effect of tetracaine and procaine on skinned muscle fibers depend on free calcium. *J. Muscle Res. Cell. Motil.* **10:**337–349
33. Ritchie, J.M., Greengard, P. 1966. On the mode of action of local anaesthetics. *Annu. Rev. Pharmacol.* **6:**405–430
34. Saida, K., Suzuki, A. 1981. Mode of action of prilocaine on sarcoplasmic reticulum in skinned skeletal muscle fibers. *J. Pharm. Exp. Ther.* **219:**815–820
35. Saito, A., Seiler, S., Chu, A., Fleischer, S. 1984. Preparation and morphology of sarcoplasmic reticulum terminal cisternae from rabbit skeletal muscle. *J. Cell Biol.* **99:**875–885
36. Schwartz, W., Palade, P.T., Hille, B. 1977. Local anaesthetics: effect of pH on use-dependent block of sodium channels in frog muscle. *Biophys. J.* **20:**343–368
37. Shoshan-Barmatz, V. 1988. ATP-dependent interaction of propranolol and local anaesthetic with sarcoplasmic reticulum. *Biochem. J.* **256:**733–739
38. Shoshan-Barmatz, V., Zarka, A. 1992. A simple, fast, one step method for the purification of skeletal muscle ryanodine receptor. *Biochem. J.* **285:**61–66
39. Smith, I.C.P., Auger, M., Jarrell, H.C. 1991. Molecular details of anaesthetic-lipid interaction. *In*: Annals, New York Academy of Sciences. E. Rubin, K.W. Miller and S.H. Roth, editors. Vol. **625:**pp. 668–684
40. Somlyo, A.P. 1985. The messenger across the gaps. *Nature* **316:**298–299
41. Suko, J., Winkler, F., Scharinger, B., Hellman, G. 1976. Aspects of the mechanism of action of local anaesthetics on sarcoplasmic reticulum of skeletal muscle. *Biochim. Biophys. Acta* **443:**571–586
42. Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Ninamino, N., Matsuo, H., Ueda, M., Hanoaka, M., Hirose, T., Numa, S. 1989. Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. *Nature* **339:**439–445
43. Volpe, P., Palade, P., Castello, B., Mitchell, R.D., Fleischer, S. 1983. Spontaneous calcium release from sarcoplasmic reticulum: Effect of local anaesthetics. *J. Biol. Chem.* **258:**12434–12442
44. Weiland, G.A., Molinoff, P.B. 1981. Quantitative analysis of drug receptor interaction: I. Determination of kinetic and equilibrium properties. *Life Sci.* **29:**313–330
45. Williams, N.F., Coleman, P.S. 1982. Exploring the adenine nucleotide binding sites on mitochondrial F_1-ATPase with a new photoaffinity probe, 3′-0-(4-Benzoyl) benzoyl adenosine 5′-triphosphate. *J. Biol. Chem.* **257:**2834–2841
46. Yagi, S., Endo, M. 1980. Effect of dibucaine on skinned skeletal muscle fibers. An example of multiple action of a drug on a single subcellular structure. *Biomedical Res. (Tokyo)* **1:**269–272
47. Zarka, A., Shoshan-Barmatz, V., 1993. Characterization and photoaffinity labeling of the ATP binding site of the ryanodine receptor from skeletal muscle. *Eur. J. Biochem. (in press)*
48. Zorzato, F., Fuyjii, J., Otsu, K., Phillips, M., Green, N.M., Lai, F.A., Meissner, G., MacLennan, D.H. 1990. Molecular cloning of cDNA encoding human and rabbit forms of Ca^{2+} release channel (Ry-Rec) of skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* **265:**2244–2256

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