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Spontaneous Calcium Release from Sarcoplasmic Reticulum

EFFECT OF LOCAL ANESTHETICS*

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Pompeo Volpe‡, Philip Palade§, Brian Costello¶, Robert D. Mitchell‖, and Sidney Fleischer**

From the Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37235

Spontaneous calcium release from purified light sarcoplasmic reticulum has been previously described (Palade, P., Mitchell, R. D., and Fleischer, S. (1983) *J. Biol. Chem.* 258, 8098–8107) and found to be distinct from several other forms of Ca^{2+} release. Ca^{2+} release occurs after a lag period following active Ca^{2+} preloading and depletion of extravesicular Ca^{2+}. In the present study, we find that local anesthetics inhibit spontaneous Ca^{2+} release, in a time-dependent manner, varying considerably in the preincubation time required to exert maximal effect. At pH 7.0, hydrophilic and mostly charged local anesthetics, such as procaine, procainamide, and *N*-(2,6-dimethylphenyl carbamoyl methyl)triethyl ammonium bromide, inhibit Ca^{2+} release only after long preincubations (hours), whereas more hydrophobic local anesthetics are effective after only a short incubation (minutes) with sarcoplasmic reticulum. The more hydrophobic anesthetics take somewhat longer to reach equilibrium, as studied by inhibition of unidirectional Ca^{2+} efflux, and there is a direct relationship between hydrophobic partition coefficient and half-time to reach equilibrium. Agents known to inhibit permeability pathways for monovalent cations *i.e.* K^+ channel blockers (decamethonium and *n*-dodecane-1,12-*N,N,N,N',N',N'*-hexamethyl-bis-ammonium) or the anion blocker (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid), do not inhibit spontaneous Ca^{2+} release. Carbonyl cyanide *m*-fluorophenylhydrazone, a protonophore, and gramicidin D, a monovalent cation ionophore, have no effect on Ca^{2+} release whether local anesthetics are present or not, while the Ca^{2+} ionophore A23187 relieves inhibition of Ca^{2+} release by local anesthetics. Ruthenium red does not inhibit spontaneous Ca^{2+} release.

These findings suggest that the binding site(s) for local anesthetics is located on the inner face of the sarcoplasmic reticulum membrane and that local anesthetics interact directly with a Ca^{2+} channel rather than with other permeability pathways which might indirectly influence Ca^{2+} channel gating.

Local anesthetics have been used for more than half a century (1) to study and influence skeletal muscle contraction. Extensive studies have been carried out with anesthetics on a variety of membrane systems including sarcoplasmic reticulum. Local anesthetics have been reported to both block (2–28) and enhance (5, 11, 12, 19, 29–40) Ca^{2+} release from SR.[1] Reasons for such divergent results include variations in (*a*) the chemical structure (5) of the anesthetics; (*b*) the concentration used (10, 11, 19, 34, 39); (*c*) the assay conditions (pH in particular *cf.* Refs. 5, 8, 20, 36); (*d*) the experimental system *e.g.* bundles of muscle fibers (1, 4–7, 16–18, 21–27, 32, 37, 38), skinned fibers (12–15, 28, 33), or isolated sarcoplasmic reticulum (2, 3, 8–11, 19, 20, 29–31, 34–36); (*e*) the source of isolated SR *i.e.* frog *versus* rabbit (2, 3, 9); and (*f*) the postulated trigger mechanism for Ca^{2+} release *i.e.* Ca^{2+}-induced (8, 20, 41), caffeine-induced (5, 6, 9, 25, 26, 32, 33), or spontaneous (11). Depolarization-induced Ca^{2+} release *i.e.* Ca^{2+} release elicited by substituting an impermeant anion with a permeant one, was not affected by procaine using either skinned fibers (13, 41) or isolated SR (42).

As reported recently (43),[2] spontaneous Ca^{2+} release[3] requires a critical extent of calcium preloading in the presence of high phosphate levels and takes place, after a lag period, when the free $[Ca]_0$ has been reduced to the submicromolar range. The experiments presented here deal with the influence of local anesthetics on spontaneous Ca^{2+} release from skeletal SR. The data are consistent with the existence of a Ca^{2+} efflux pathway (Ca^{2+} channel) which is selectively blocked by local anesthetics from the inner face of the SR membrane. A preliminary report has appeared (44).

* This is the third paper in a series on "Spontaneous Calcium Release from Sarcoplasmic Reticulum." The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Postdoctoral Fellow of Muscular Dystrophy Association. To whom correspondence should be addressed at, Department of Physiology, UMDNJ New Jersey Medical School, 100 Bergen Street, Newark, NJ 07103.

§ Postdoctoral Fellow under Public Health Service Grant 5F32 AM 06386-02. Present address, Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, TX 77550.

¶ Postdoctoral Fellow under National Institutes of Health Grant 5F32 GM 08198-01.

‖ Postdoctoral Fellow of Muscular Dystrophy Association. Present Address, Roche Institute of Molecular Biology, Nutley, NJ 07110.

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[1] The abbreviations used are: SR, sarcoplasmic reticulum; light and heavy SR, are referable to longitudinal and terminal cisternal portion of sarcoplasmic reticulum, respectively; FCCP, carbonyl cyanide *m*-fluorophenylhydrazone; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; bis-Q10, decamethonium; SKF 525-A, β-diethylaminoethyl diphenylpropylacetate-HCl; nifedipine, 2,6-dimethyl-3,5-dicarbomethoxy-4-(2-nitro)phenyl-1,4-dihydropyridine; QX 314, *N*-(2,6-dimethylphenylcarbamoylmethyl)triethyl ammonium bromide; QX 572, *N,N*-bis(phenylcarbamoylmethyl)dimethyl ammonium chloride; bis-Q12, *n*-dodecane-1,12-*N,N,N,N',N',N'*-hexamethyl-bis-ammonium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl-ether)-*N,N,N',N'*-tetraacetic acid; *F*, hydrophobic partition coefficient; $pCa_0 = -\log_{10}$ (external calcium ion concentration).

[2] R. D. Mitchell, P. Palade, and S. Fleischer, submitted to *J. Biol. Chem.*

[3] Throughout this paper, *Ca^{2+} release* is used to refer to net loss of calcium by SR vesicles and *Ca^{2+} efflux* refers to unidirectional calcium efflux from the SR vesicles.

EXPERIMENTAL PROCEDURES

Materials—Procaine HCl, tetracaine HCL, dibucaine HCl, procainamide HCl, benzocaine, antipyrylazo III, arsenazo III, creatine phosphokinase, Na_2 phosphocreatine, FCCP, gramicidin D, ruthenium red, DIDS, bis-Q10, and quercetin were obtained from Sigma (St. Louis, MO). A23187 was obtained from Calbiochem (LaJolla, CA). Ethyl-*o*-toluate was obtained from ICN Pharmaceuticals (Plainview, NY). SKF 525-A was a gift from SmithKline and French Laboratories (Philadelphia, PA), and nifedipine from Delbay Pharmaceuticals (Bloomfield, NJ). Prilocaine-HCl, mepivacaine-HCl, bupivacaine-HCl, etidocaine-HCl, lidocaine-HCl, and its quaternary derivatives QX 314 and QX 572 were a gift from Astra Pharmaceutical Products (Worcester, MA). Bis-Q12 was the gift of Dr. Christopher Miller (Brandeis University, Waltham, MA). $^{45}CaCl_2$ was obtained from New England Nuclear (Boston, MA). All other chemicals were of the highest purity available. Lidocaine, dibucaine, etidocaine, bupivacaine, benzocaine, FCCP, nifedipine, A23187, gramicidin D, bis-Q10, bis-Q12, and quercetin were prepared in concentrated ethanolic solutions. Tetracaine, procaine, prilocaine, mepivacaine, procainamide, QX 314, QX 572, and DIDS were dissolved in the assay buffer, 125 mM Na- or K phosphate, pH 7.0. SKF 525-A in ethanolic or aqueous solution gave similar results.

Physical and Chemical Properties of Local Anesthetics—The chemical structures of the local anesthetics used in the present study are shown in Fig. 1. Three categories of local anesthetics were used. (Some physical and chemical properties are summarized in Table I.) (*a*) Tertiary amines were SKF 525-A (40), tetracaine, prilocaine, mepivacaine, dibucaine, bupivacaine, etidocaine, lidocaine, procaine, and procainamide. At pH 7.0, which is below their pK_a values, these compounds are predominantly protonated and, therefore, in their charged, cationic form. The hydrophobic partition coefficient (F) for the free base form differs considerably among the drugs of this class. The calculated effective distribution coefficient at pH 7.0 ($q_{7.0}$), which relates the [base] in hydrophobic phase to [base] and [cation] in aqueous phase (see legend to Table I), is considered to be a more reliable index of the ability of these compounds to cross membranes (45). (*b*) Neutral anesthetics were benzocaine and ethyl-*o*-toluate. (*c*) Quaternary amines, QX 572 and QX 314, are permanently in a charged, cationic form. QX 572 is much more hydrophobic than QX 314 with an estimated permeability coefficient of 10^{-4} cm/s, as compared with 2×10^{-8} cm/s for QX 314 (45).

Preparation of Isolated SR and Preincubation with Local Anesthetics—Light SR, derived mostly from longitudinal sarcoplasmic reticulum of rabbit fast-twitch skeletal muscle, was isolated and purified as previously described (46). SR vesicles in 10% sucrose, 5 mM K Hepes, pH 7.2, were stored in small aliquots at −70 °C until used. The biochemical characteristics of this SR fraction were described in Mitchell *et al.* (46) and in the first paper of this series (43).

Preincubation of SR with local anesthetics was carried out at room temperature (23–24 °C) in the assay buffer, either Na- or K phosphate, pH 7.0, as detailed in the figure legends. Shortly before commencing assay, other ingredients were added (see below) and the reaction was started with addition of 1 mM Na_2 ATP.

Ca^{2+} Loading and Ca^{2+} Release Assay—Ca^{2+} loading and Ca^{2+} release were measured, as previously described (43), at 25 °C in a medium containing, in a final volume of 1 ml, 112.5 mM Na phosphate (Na medium) or K phosphate (K medium), pH 7.0, 1 mM $MgSO_4$, 1 mM Na_2 ATP, 5 mM Na_2 phosphocreatine, 20 μg of creatine phosphokinase, and either 200 μM antipyrylazo III or 20 μM arsenazo III. The reaction was started by adding 20–30 μg of SR protein. The loading of 3–4 sequentially added 50-nmol $CaCl_2$ pulses was monitored by following differential absorbance changes of antipyrylazo III at 710–790 nm or of arsenazo III at 660–740 nm in a Hewlett-Packard 8450 A spectrophotometer. Antipyrylazo III was used in most of the experiments because it gives better signal-to-noise characteristics and somewhat higher Ca^{2+} loading rates than arsenazo III (43). The extent and rate of spontaneous Ca^{2+} release were determined with respect to the absorbance change brought about by the last Ca^{2+} pulse. When a drug was administered after the completion of Ca^{2+} loading but before release, a recalibration with Ca^{2+} was required. In the experiments carried out with QX 314 and QX 572, Ca^{2+} fluxes were followed using only arsenazo III because of the interaction between these drugs and antipyrylazo III. When ethanolic solutions of drugs were used, equal amounts of alcohol were added to the control (0.5–1%).

Ca^{2+} Efflux Assays—Unidirectional Ca^{2+} efflux[3] was measured in

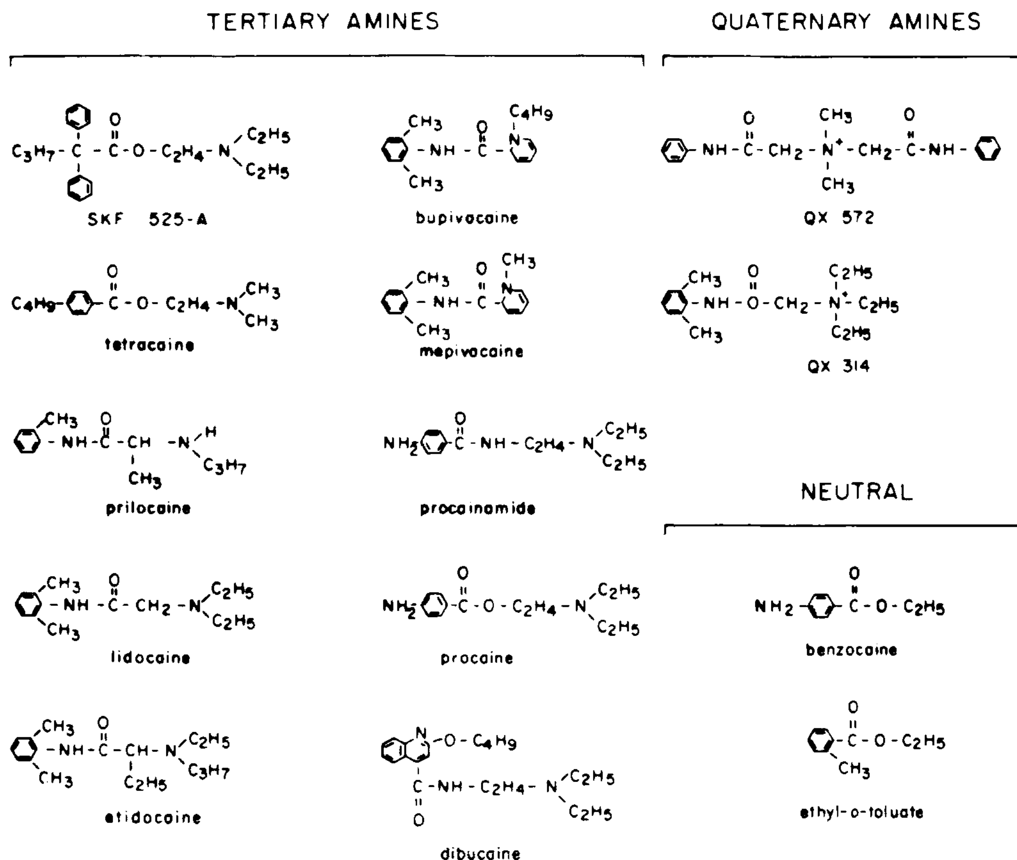

FIG. 1. **Chemical structures of the local anesthetics used in this study.** Chemical and physical characteristics are summarized in Table I.

TABLE I

Chemical and physical properties of the local anesthetics used in this study

The chemical structures of the local anesthetics are given in Fig. 1. References for pK_a values are SmithKline and French Laboratories for SKF 525-A, Büchi *et al.* (72) for benzocaine, and Courtney (60) for all the others. *F* is the octanol/water (or buffer) hydrophobic partition coefficient for the free base form. The values are derived from Courtney (60) except for benzocaine, dibucaine, and SKF 525-A (Leo *et al.* (73)). For QX 572 and QX 314, the oleyl alcohol/phosphate buffer partition coefficient is derived from Hille (45). $q_{7.0}$ is the effective distribution coefficient of [base] in the hydrophobic phase *versus* [base] plus [cation] in buffer at pH 7.0 and is calculated from $q_{pH} = F/(1 + 10^{(pK_a - pH)})$. The percentage of free base form (*A*) in the medium at pH 7.0 is calculated from the Henderson-Hasselbalch equation *i.e.* $\log([AH^+]/[A]) = pK_a - pH$.

	M_r	pK_a	F	$q_{7.0}$	% free base (pH 7.0)
Tertiary amines					
SKF 525-A	353	8.8[a]	44,668	697	1.56
Tetracaine	265	8.5	2,512	77.02	3.07
Etidocaine	276	7.7	23,998	3,992	16.63
Bupivacaine	302	8.1	21,379	1,573	7.35
Prilocaine	220	7.8	60.25	8.24	13.68
Lidocaine	234	7.9	575	64.30	11.19
Mepivacaine	246	7.6	109.64	22.01	20.08
Procaine	236	8.9	100	1.24	1.24
Procainamide	235	9.2	5.6	0.04	0.63
Dibucaine	344	8.5	5,000	153.30	3.07
Neutral					
Benzocaine	165	2.6	41	41	100.00
Quaternary amines					
QX 572	312		0.9		0.00
QX 314	263		Very low		0.00

[a] The pK_a value reported by Suarez-Kurtz and Bianchi (40) *i.e.* 5.7, is at variance with that measured by SmithKline and French Laboratories *i.e.* 8.8.

the presence of either quercetin or EGTA to inhibit the Ca^{2+} pump. The quercetin method, involving only spectrophotometric assay, was more convenient than the EGTA method.

In Method A, Ca^{2+} efflux was measured by adding 100 μM quercetin (5 μl of 20 mM stock solution) after the completion of Ca^{2+} loading, at the beginning of the lag period. The assay medium was the same as that described above. A final pulse of Ca^{2+} was added at the end of each experiment in order to recalibrate the absorbance changes of the Ca^{2+} indicator (see the shift in the base line, *inset* of Fig. 7). Quercetin is known to inhibit the forward and backward reaction of the Ca^{2+} pump (47) and not to inhibit Ca^{2+} release (43, 47). 100 μM quercetin inhibited Ca^{2+} loading almost completely and Ca^{2+} reuptake after spontaneous Ca^{2+} release completely (see *inset* of Fig. 7). Quercetin did not change the length of the lag phase preceding spontaneous Ca^{2+} release. The rate and extent of Ca^{2+} efflux, as well as inhibition by local anesthetics, measured using quercetin were in good agreement with those measured using EGTA and $^{45}CaCl_2$ (see Method B and legend to Fig. 7). Thus, under the prevailing experimental conditions, quercetin does not enhance unidirectional spontaneous Ca^{2+} efflux from purified light SR (Fig. 9C in Ref. 43).

In Method B, Ca^{2+} efflux was also measured by means of isotope techniques as described previously (43). The assay medium was similar to that described above except for the deletion of quercetin and the final volume was 2 ml. A small aliquot of a high specific activity $^{45}CaCl_2$ was added before the loading was started. The loading of 300 nmol of $CaCl_2$ by 45-60 μg of SR protein was monitored following the absorbance changes of either antipyrylazo III or arsenazo III. At the completion of the loading, 1 mM EGTA was added (estimated resulting free $[Ca]_o = 3 \times 10^{-8}$ M), and 0.1-ml aliquots were withdrawn over the next few minutes. Each aliquot was rapidly filtered using a microfilter kit and 0.2 μm nitrocellulose filters (Schleicher and Schuell, Keene, NH). Filtrates were counted to monitor isotope released from the SR vesicles.

In Method C, Ca^{2+} efflux at fixed free $[Ca]_o$ was determined by means of isotope techniques as described above (Method B). At the completion of Ca^{2+} loading, 40 μl of concentrated Ca-EGTA solution (250 mM EGTA) was added to the standard assay medium (2 ml) so that free $[Ca]_o$, during Ca^{2+} efflux, was kept constant at either 10^{-8} or 10^{-4} M. Aliquots (0.1 ml) were withdrawn and filtered and filtrates counted for radioactivity. Ca-EGTA solutions were prepared with the aid of a computer program (43) using the association constants and calculations reported by Fabiato and Fabiato (48).

RESULTS

The effect of three categories of local anesthetics on spontaneous calcium release from sarcoplasmic reticulum was studied. In the range of anesthetic concentrations used, Ca^{2+} release was selectively inhibited *i.e.* Ca^{2+} loading was affected only slightly, or not at all (Table II).

Tertiary Amine Local Anesthetics—A typical control experiment illustrating spontaneous Ca^{2+} release[3] is shown in Fig. 2*a*. After contaminating Ca^{2+} is sequestered by SR (*arrowhead*), three consecutive 50-nmol $CaCl_2$ pulses were administered (*arrows*). Spontaneous Ca^{2+} release occurred at the completion of Ca^{2+} loading, after a lag period of about 75 s, followed by Ca^{2+} reuptake.

Increasing concentrations of lidocaine lead to progressive inhibition of Ca^{2+} release (Fig. 2, *b–d*). Lidocaine was administered just prior to the first $CaCl_2$ pulse (*L* in Fig. 2), 5–6 min before the onset of spontaneous release. At lidocaine concentrations of 6, 9, and 15 mM, the Ca^{2+} release rate decreased approximately 15, 40, and 84%, respectively.

Preincubation of SR with the anesthetics enhanced their inhibitory action on Ca^{2+} release (Fig. 3). The onset of the action of lidocaine was relatively rapid, but some anesthetics were much slower in taking effect. Procainamide and procaine, both less hydrophobic than lidocaine and mostly in the charged form (Table I), required long preincubations with SR in order to inhibit Ca^{2+} release. After 4 h of preincubation at room temperature, procainamide or procaine (10 mM of each)

TABLE II

Effect of local anesthetics on Ca^{2+} loading and Ca^{2+} release rates

Ca^{2+} loading and Ca^{2+} release were measured as described under "Experimental Procedures." SR vesicles were loaded with 5.4–6.8 μmol of Ca^{2+}/mg of protein. K_i is defined as the concentration of local anesthetic which inhibited the Ca^{2+} release rate by 50% when added just prior to initiation of Ca^{2+} loading, and was calculated from a linear plot of % inhibition of control activity *versus* anesthetic concentration. Ca^{2+} loading rates were measured at K_i concentration of anesthetic. The values are given as the mean ± S.D. for the number of different SR preparations shown in parentheses. Some anesthetics required prolonged preincubation for inhibition. For example, Ca^{2+} release was inhibited 50% after 2-h incubation with 10 mM procaine (3 experiments, see also Fig. 3) and after 1.5-h incubation with 5 mM QX 314 (2 experiments, see Fig. 6).

	K_i	Ca^{2+} loading rates at K_i (% of control)
	mM	
Tertiary amines[a]		
SKF 525-A	0.047 ± 0.011 (4)	90.1 ± 3.5 (4)
Tetracaine	0.49 ± 0.06 (3)	93.9 ± 3.3 (3)
Etidocaine	2.07 ± 0.33 (3)	100 (3)
Bupivacaine	2.66 ± 0.03 (3)	100 (3)
Prilocaine	11.38 ± 1.13 (3)	91.8 ± 4.1 (3)
Lidocaine	10.94 ± 0.89 (4)	97.0 ± 2.6 (4)
Mepivacaine	12.53 ± 1.28 (3)	92.3 ± 2.0 (3)
Neutral		
Benzocaine	4.00 ± 0.56 (4)	100 (4)
Quaternary amines		
QX 572	0.19 ± 0.03 (4)	84.0 ± 6.2 (4)

[a] In preliminary experiments, dibucaine was found to markedly inhibit Ca^{2+} loading rate. Whether this finding was due to inhibition of Ca^{2+} loading activity (29, 31, 34, 35, 71) or increase of Ca^{2+} permeability (11, 12) was not further investigated. Nonetheless, when dibucaine (0.5–1 mM) was added after Ca^{2+} loading, the rate of Ca^{2+} release was decreased (by 35%) but the extent of release was increased (1.8 times).

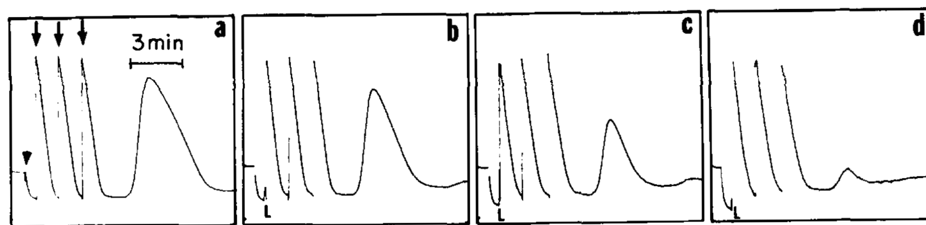

FIG. 2. **Inhibition of Ca^{2+} release by lidocaine.** Ca^{2+} loading and Ca^{2+} release were measured as described under "Experimental Procedures" in K medium, using antipyrylazo III as the calcium indicator. SR (23 μg of protein; *arrowhead*) was first added, then three consecutive 50-nmol $CaCl_2$ pulses (*arrows*) were administered. Spontaneous Ca^{2+} release was observed after a lag period subsequent to removal of the third pulse of $CaCl_2$ from the medium. In *b*, *c*, and *d*, lidocaine (*L*) was added prior to the first $CaCl_2$ pulse. *a*, Control trace (2.85 μmol of Ca^{2+} released/min·mg of protein); *b*, *c*, and *d*, additions of 6, 9, and 15 mM lidocaine, reduced the rate of Ca^{2+} release by 15, 40, and 84%, respectively.

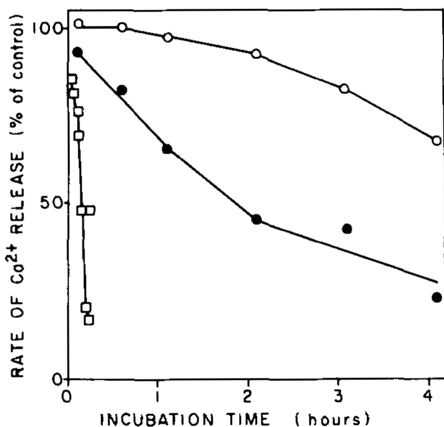

FIG. 3. **Time dependence of inhibition of Ca^{2+} release by lidocaine, procaine, and procainamide.** Ca^{2+} loading and Ca^{2+} release were measured in Na medium. SR (28 μg of protein) was loaded with $CaCl_2$ administered in three 50-nmol pulses. In the case of procaine (●, 10 mM) and procainamide (○, 10 mM), the first time point was obtained by adding the local anesthetic prior to Ca^{2+} loading (see legend to Fig. 2). Subsequent time points were obtained after preincubation (see "Experimental Procedures"), as indicated on the *abscissa*. Incubation time is the effective interaction time between SR and local anesthetics before Ca^{2+} release takes place. In the case of lidocaine (□, 10 mM), the first four time points were obtained by adding the local anesthetic after uptake of the third Ca^{2+} addition, before the third Ca^{2+} addition, before the second Ca^{2+} addition, and before the first Ca^{2+} addition, respectively. Other time points were obtained after short preincubations up to 15 min. Rates of Ca^{2+} release are expressed as percentage of activity of the control which was incubated without anesthetic for the same length of time. The average control rate of Ca^{2+} release was 2.80 μmol of Ca^{2+}/min·mg of protein and decreased by approximately 15% after prolonged incubations (3-4 h).

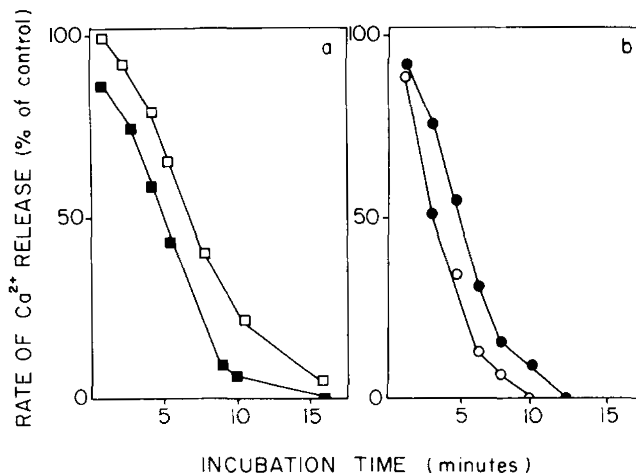

FIG. 4. **Time dependence of inhibition of Ca^{2+} release by tetracaine and SKF 525-A.** Ca^{2+} loading and Ca^{2+} release were measured in K medium. SR (28 μg of protein) was loaded with $CaCl_2$ administered in 3 consecutive 50-nmol pulses. The first four time points were obtained by adding the local anesthetic after uptake of the third Ca^{2+} addition, before the third Ca^{2+} addition, before the second Ca^{2+} addition, and before the first Ca^{2+} addition, respectively. Subsequent time points were obtained after short preincubations, as indicated on the *abscissa*. Rates of Ca^{2+} release are expressed as percentage of activity of the control incubated without anesthetic for the same length of time. *a*, tetracaine, □, 0.45 mM; ■, 0.6 mM. *b*, SKF 525-A, ●, 50 μM; ○, 75 μM.

decreased the rate of Ca^{2+} release by 35 and 75%, respectively (Fig. 3).

Tetracaine and SKF 525-A, both hydrophobic and mostly charged (Table I), were found to inhibit Ca^{2+} release when administered just prior to Ca^{2+} loading. Under these conditions, the rate of Ca^{2+} release was inhibited by 50% with either 0.49 mM tetracaine or 47 μM SKF 525-A (Table II). The inhibition of Ca^{2+} release by tetracaine (Fig. 4*a*) or SKF 525-A (Fig. 4*b*) was sharply time-dependent. The first time points in Fig. 4 were obtained by adding anesthetics after the completion of Ca^{2+} loading so that the inhibitory effect was more marked after a few minutes of preincubation.

Etidocaine, bupivacaine, prilocaine, and mepivacaine inhibited Ca^{2+} release when administered in the millimolar range just prior to Ca^{2+} loading (Table II). Prilocaine, which is less hydrophobic but with a higher percentage of free base form than procaine (Table I), was found to be effective without a long preincubation period, whereas procaine required prolonged preincubation to be effective. The onset of action of tertiary amines varies with both hydrophobicity (*F*) and the percentage of free base form (see Table I).

Neutral Local Anesthetics—Benzocaine in millimolar concentration inhibited Ca^{2+} release. When benzocaine was added just prior to Ca^{2+} loading, 50% inhibition was obtained at 4 mM (Table II). Benzocaine also exhibited a sharp time-dependence for its inhibition (data not shown). In the same range of concentration, ethyl-*o*-toluate, a steric analog of benzocaine (17), was found to be ineffective (data not shown).

Quaternary Amine Local Anesthetics—QX 572, which is membrane-permeant despite being permanently charged (45, 49) (Table I), was found to inhibit Ca^{2+} release when administered in the submillimolar range just prior to Ca^{2+} loading (Fig. 5, *b-d* and Table II). QX 572 also decreased the rate of Ca^{2+} loading more than the other anesthetics used in this study (see Table II), and when added after the completion of Ca^{2+} loading (Fig. 6, first time point), it enhanced rather than inhibited Ca^{2+} release.

QX 314, which like procaine and procainamide is poorly membrane-permeant, was found to be a much less effective inhibitor of spontaneous calcium release unless a long preincubation was performed. Fig. 6 shows that QX 572 at 0.25

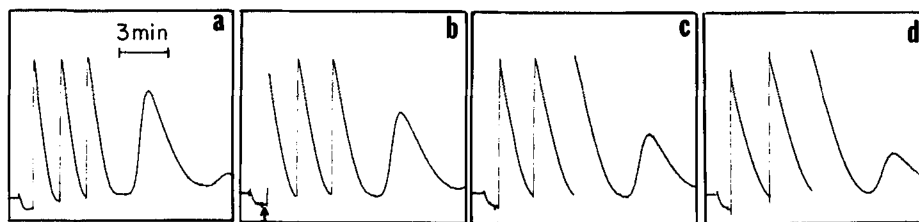

FIG. 5. **Inhibition of Ca^{2+} release by QX 572.** Ca^{2+} loading and Ca^{2+} release were measured in Na medium using arsenazo III as the calcium indicator. SR (23 μg of protein) was added to the assay medium, followed by three consecutive 50-nmol $CaCl_2$ pulses. *a*, Control trace (2.4 μmol of Ca^{2+} released/min·mg of protein); *b*, *c*, and *d*, 100, 200, and 300 μM QX 572, respectively. QX 572 was added before Ca^{2+} loading (*arrow*). In this experiment, the Ca^{2+} loading rate was decreased to 78% of the control with 200 μM QX 572 (*c* and Table II), the concentration which inhibited the Ca^{2+} release rate by 50%.

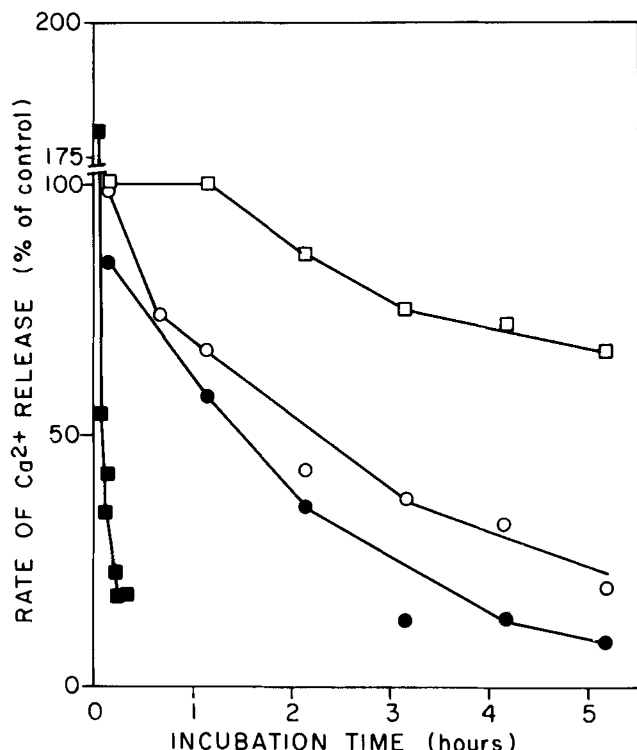

FIG. 6. **Time dependence of inhibition of Ca^{2+} release by QX 572 and QX 314.** Ca^{2+} loading and Ca^{2+} release were measured in Na medium using arsenazo III as the calcium indicator. SR (28 μg of protein) was loaded with three 50-nmol $CaCl_2$ pulses. In the case of QX 572 (■, 0.25 mM), the first four time points were obtained by adding the local anesthetic after uptake of the third Ca^{2+} addition, before the third Ca^{2+} addition, before the second Ca^{2+} addition, and before the first Ca^{2+} addition, respectively. For QX 314 (□, 0.25 mM; ○, 2.5 mM; ●, 5 mM), the first time point was obtained by adding the local anesthetic prior to Ca^{2+} loading. All other time points were obtained after preincubation, as indicated on the *abscissa.*

mM strongly decreased Ca^{2+} release within a few minutes of preincubation, whereas 0.25 mM QX 314 inhibited the rate of Ca^{2+} release only 30% after 5 h of preincubation. Higher concentrations of QX 314 were more effective (Fig. 6).

Time Dependence of Inhibition of Ca^{2+} Efflux by Local Anesthetics—The inhibition of Ca^{2+} release was found to be time-dependent (Figs. 3, 4, and 6). This finding would suggest that local anesthetics must cross a hydrophobic barrier to exert their inhibitory action on spontaneous Ca^{2+} release from SR. Thus, an attempt was made to discriminate between equilibrium and rate effects *i.e.* to distinguish factors related to anesthetic binding to a site from those pertinent to reaching the site. A Ca^{2+} efflux assay was utilized, instead of the Ca^{2+} release assay, thereby measuring unidirectional Ca^{2+} efflux and avoiding interference by Ca^{2+} pumping activity. As observed in Fig. 4, Ca^{2+} release may disappear when Ca^{2+} pump-mediated Ca^{2+} influx exceeds a partially inhibited Ca^{2+} efflux. Under such circumstances, an equilibrium might be erroneously assumed, leading to an underestimate of the time to attain equilibrium.

Unidirectional Ca^{2+} efflux[3] was measured by inhibiting the Ca^{2+} pump activity with 100 μM quercetin after the completion of Ca^{2+} loading (see *inset* in Fig. 7). The time dependence of inhibition by each local anesthetic on Ca^{2+} efflux was examined at several concentrations, and the IC_{50} *i.e.* the concentration which at equilibrium inhibited Ca^{2+} efflux by 50% (Fig. 7 and Table III) was determined. QX 572,[4] which is less lipid soluble than SKF 525-A, reached equilibrium approximately three times faster. Equilibration times for benzocaine (Fig. 7 and Table III), prilocaine, mepivacaine, lidocaine, tetracaine, bupivacaine, and etidocaine (Table III) were intermediate. More hydrophobic local anesthetics took longer to reach equilibrium and the half-time to reach equilibrium could be correlated with the logarithm of the hydrophobic partition coefficient (Fig. 8a). Three anesthetics did not conform to this trend requiring very long times to approach equilibrium. Procaine and procainamide (10 mM of each drug) and QX 314 (2.5 mM), after 4 h of preincubation, decreased Ca^{2+} efflux by 45, 20, and 55%, respectively, without reaching equilibrium (data not shown).

At equilibrium, SKF 525-A was the most effective local anesthetic for inhibiting Ca^{2+} efflux. QX 572 and tetracaine also were effective in the submillimolar range. Equilibrium potency for the other local anesthetics tested exceeded the millimolar range (Table III). The equilibrium potency (IC_{50}) did not correlate well with the hydrophobic partition coefficient (Fig. 8*b*).

pH Dependence of Inhibition of Ca^{2+} Efflux by Local Anesthetics—Since the distribution of tertiary amines across membrane is a function of [base] (45, 50, 51), we investigated the pH dependence of inhibition of Ca^{2+} efflux by local anesthetics. The rate of Ca^{2+} efflux in the absence of local anesthetics (not shown) displayed a maximum around pH 7.0, decreasing steadily at both higher and lower pH. Procaine and tetracaine, when added to the assay medium, were least inhibitory at pH 7.0 and more effective both below and above pH 7.0 (Fig. 9*a*). However, QX 314 and QX 572, both permanently charged, and benzocaine, completely in the neutral form, were insensitive to pH changes above pH 7.0 (Fig. 9*b*). QX 572 at 0.25 mM inhibited Ca^{2+} efflux by 50% above neutrality, but was more potent at lower pH. At pH 6.6, there was complete inhibition. QX 314 at 5 mM did not affect Ca^{2+}

[4] QX 572, when added after completion of Ca^{2+} loading (first time point in Fig. 7), does not increase Ca^{2+} efflux, suggesting that the enhancement of Ca^{2+} release (first time point in Fig. 6) is due to a distinct, inhibitory effect on the Ca^{2+} pump activity.

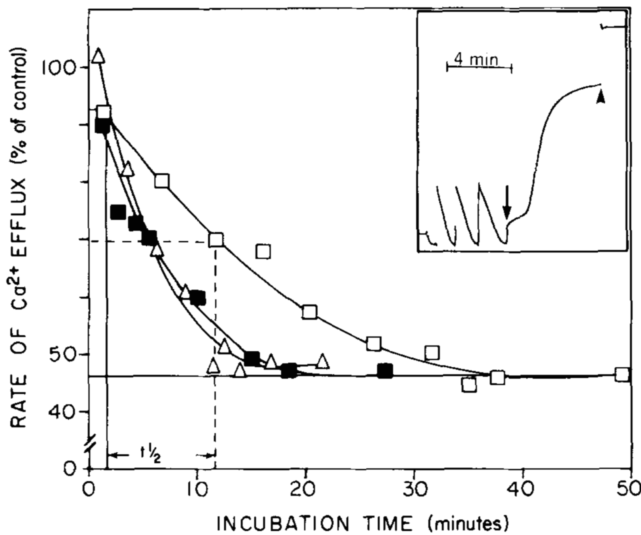

FIG. 7. **Time dependence of inhibition of Ca^{2+} efflux by local anesthetics.** Ca^{2+} efflux was measured, as described under "Experimental Procedures," in K medium by adding 100 μM quercetin at the completion of Ca^{2+} loading (*arrow in the inset*). All experiments were performed on the same SR preparation, using the same amount of SR protein (24 μg) and the same level of Ca^{2+} preloading (6.25 μmol of Ca^{2+}/mg of protein). Arsenazo III (△) or antipyrylazo III (□, ■, and *inset*) were used as calcium indicators. *Inset* shows a typical trace of a control experiment; the *arrow* indicates addition of 100 μM quercetin. The rate of Ca^{2+} efflux was 4.14 μmol of Ca^{2+}/min·mg of protein. The *arrowhead* indicates the addition of 50 nmol of $CaCl_2$ for recalibration of the calcium indicator. The rate of Ca^{2+} efflux measured on the same sample by means of isotope techniques, in the presence of 1 mM EGTA (see "Experimental Procedures") was 4.05 μmol of Ca^{2+}/min·mg of protein. 0.2 mM QX 572 (△), 6 mM benzocaine (■), and 40 μM SKF 525-A (□) were used at concentrations which at equilibrium inhibited the rate of Ca^{2+} efflux by 50%. The curves were drawn by eye. The determination of the half-time to reach equilibrium ($t_{½}$) for SKF 525-A is drawn. The effect of local anesthetics on Ca^{2+} efflux, using either the quercetin method or isotopic technique, was essentially the same.

TABLE III

Equilibrium parameters of inhibition of Ca^{2+} efflux by local anesthetics

Experimental conditions were as in Fig. 7. Antipyrylazo III was used as the calcium indicator except for QX 572 where arsenazo III was used. IC_{50} is defined as the concentration of local anesthetic which inhibits Ca^{2+} efflux rate by 50% at equilibrium, and was determined by trial and error. The half-time to reach equilibrium was calculated as described in Fig. 7.

	Equilibrium potency IC_{50}	Half-time to reach equilibrium
	mM	*s*
Tertiary amines		
SKF 525-A	0.04	580
Tetracaine	0.45	390
Etidocaine	2.00	480
Bupivacaine	2.40	460
Prilocaine	10.00	310
Lidocaine	11.50	330
Mepivacaine	13.00	330
Neutral		
Benzocaine	6.00	290
Quaternary amines		
QX 572	0.20	200

efflux at any pH. The inhibition by benzocaine (5 mM) was virtually unaffected by pH changes.

Effect of Cation Channel Blockers and Anion Channel Blockers on Ca^{2+} Release and Efflux—Sarcoplasmic reticulum contains independent pathways for permeation of K^+ or Na^+ (52–

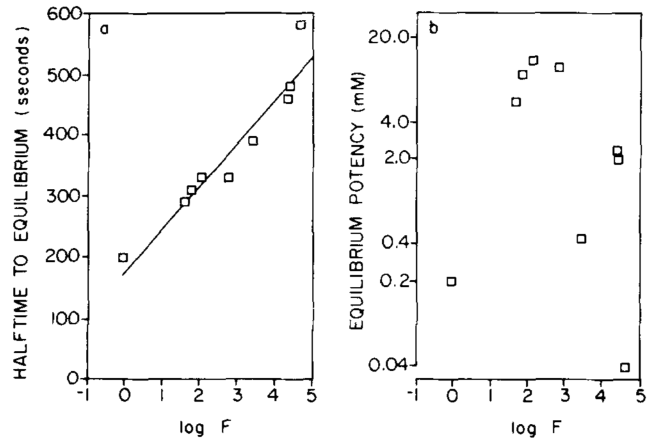

FIG. 8. **Correlation of hydrophobicity (log *F*) with half-time to reach equilibrium (*a*) and with equilibrium potency (*b*).** The half-time to reach equilibrium and the IC_{50} (concentration of local anesthetic which inhibited the rate of Ca^{2+} efflux by 50% at equilibrium) are from Table III. *F* values are from Table I. Correlation coefficients, determined by regression analysis, are 0.96 and −0.30 in *a* and *b*, respectively.

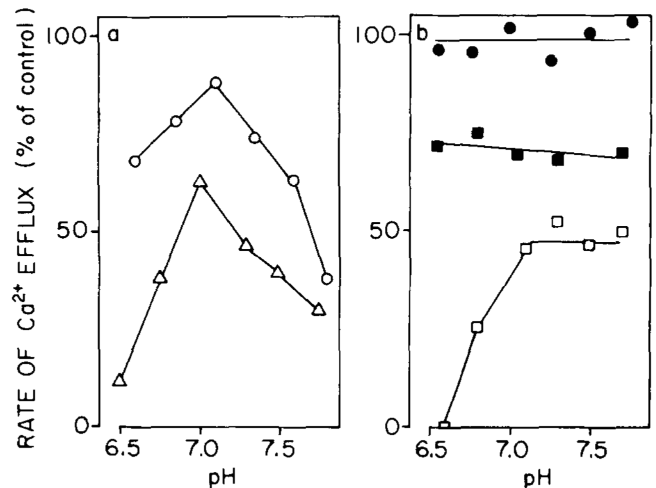

FIG. 9. **pH dependence of inhibition of Ca^{2+} efflux by local anesthetics.** Ca^{2+} efflux was measured in Na medium, as described under "Experimental Procedures," by adding 100 μM quercetin after Ca^{2+} loading. SR (28 μg of protein) was loaded with 150 nmol of $CaCl_2$ and the local anesthetics were added prior to Ca^{2+} loding. All buffer solutions contained 125 mM phosphoric acid and the pH was adjusted with NaOH. The pH values indicated on the *abscissa* were measured following each experiment. Arsenazo III (□, ●) or antipyrylazo III (■, ○, △) were used as calcium indicators. *a*, 10 mM procaine (○); 0.45 mM tetracaine (△). b, 5 mM QX 314 (●); 5 mM benzocaine (■); 0.25 mM QX 572 (□).

54), anions (55), and protons (56). Their function, *in vivo*, may be to provide an electrical shunt across the SR membrane thereby preventing charge imbalance during Ca^{2+} uptake and Ca^{2+} release (56).

Local anesthetics might conceivably exert their primary action on one or more of these permeabilities, with inhibition of Ca^{2+} release being a secondary effect. We, thus, investigated the effect on Ca^{2+} release of known inhibitors of either the anion permeability or the Na^+ and K^+ channel.

DIDS, which has been reported by Kasai (55) and Kasai and Taguchi (57) as a potent inhibitor of anion permeability of SR (K_d = 40 μM), was found to enhance Ca^{2+} release rate (Fig. 10*b*) when administered after the completion of Ca^{2+} loading (177% of the control with 200 μM). This enhancement

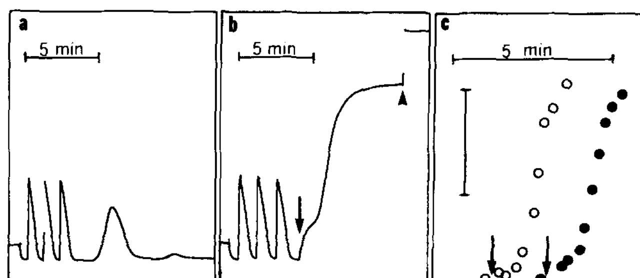

FIG. 10. **Effect of DIDS on Ca^{2+} release and Ca^{2+} efflux from SR.** Ca^{2+} loading was measured in K medium, in a final volume of 2 ml as described under "Experimental Procedures." SR (56 μg of protein) was loaded with 3 consecutive 100-nmol $CaCl_2$ pulses. Antipyrylazo III was used as the calcium indicator. *a* shows a control trace; the rate of Ca^{2+} release was 2.39 μmol of Ca^{2+}/min·mg of protein. *b* shows the trace obtained with 200 μM DIDS added during the lag phase (*arrow*); the rate of Ca^{2+} release was 4.22 μmol of Ca^{2+}/min·mg of protein. The *arrowhead* denotes the addition of 100 nmol of $CaCl_2$ for recalibration of antipyrylazo III. Ca^{2+} loading rate in the presence of 200 μM DIDS was 1% of the control activity. *c* shows Ca^{2+} efflux in the absence (○) and presence (●) of 200 μM DIDS added during the lag phase. Experimental conditions were as for *a* and *b* except that $^{45}CaCl_2$ (~10^6 cpm) was administered together with the SR. After completion of Ca^{2+} loading, 1 mM EGTA was added (*arrow*) and 0.1-ml aliquots were withdrawn and rapidly filtered (see "Experimental Procedures"). Filtrates were counted for ^{45}Ca. Ca^{2+} efflux rates were 4.16 and 3.94 μmol of Ca^{2+}/min·mg of protein in the absence and presence of DIDS, respectively. The traces are displaced with respect to each other for the sake of clarity. The *vertical bar* represents 100 nmol of $CaCl_2$.

appears to be due to inhibition of Ca^{2+} loading, since Ca^{2+} efflux was not stimulated (Fig. 10c), whereas Ca^{2+} loading and (Ca^{2+}, Mg^{2+})-ATPase activities were practically abolished by 200 DIDS[5] (see also legend to Fig. 10).

Two specific K^+ channel blockers were used *i.e.* bis-Q10 and bis-Q12 which have a blocking site on the inner face of the SR membrane (58). Neither 1–5 mM bis-Q10 nor 200 μM bis-Q12 affected Ca^{2+} release, even when long preincubations (up to 3 h) were performed (data not shown).

Effect of Ionophores on Ca^{2+} Release and Efflux—If local anesthetics were to inhibit Ca^{2+} release by blocking monovalent cation or proton permeability, addition of specific ionophores (59) should restore Ca^{2+} release. 5–10 μM FCCP, a protonophore, or 5–20 μg/ml of gramicidin D, a monovalent cation ionophore, did not change the characteristics of spontaneous Ca^{2+} release or modify Ca^{2+} efflux (data not shown). Inhibition of Ca^{2+} release by SKF 525-A (60 μM), QX 572 (0.25 mM), tetracaine (0.6 mM), or benzocaine (6 mM) was not relieved by 10 μM FCCP or 5 μg/ml of gramicidin D (data not shown). By contrast, when Ca^{2+} release was completely blocked with 100 μM SKF 525-A, subsequent addition of 1 μM A23187, a Ca^{2+} ionophore, caused rapid Ca^{2+} release (data not shown). Ca^{2+} release from SR can be restored only when an extrinsic Ca^{2+} pathway (A23187) replaces the intrinsic Ca^{2+} pathway blocked by local anesthetics.

Effect of Ruthenium Red and Nifedipine on Ca^{2+} Release—Ruthenium red (20 to 60 μM), a specific inhibitor of Ca^{2+}-induced Ca^{2+} release in heavy SR (68), and nifedipine (150 μM), a specific inhibitor of the sarcolemma Ca^{2+} channel (74), did not inhibit spontaneous Ca^{2+} release from purified light SR (data not shown). These findings suggest that: 1) spontaneous Ca^{2+} release and Ca^{2+}-induced Ca^{2+} release operate through different mechanisms; 2) ruthenium red-sensitive Ca^{2+} channels are absent in light SR (68); and 3) the Ca^{2+} pathway (Ca^{2+} channel) involved in spontaneous Ca^{2+} release has a distinct pharmacological profile.

[5] A. Chu, personal communication.

TABLE IV

Dependence of local anesthetic action upon free $[Ca]_0$

Ca^{2+} loading was measured in Na medium, in a final volume of 2 ml, in the presence of $^{45}CaCl_2$ (~10^6 cpm). All experiments were carried out on the same SR preparation, using 54 μg of protein preloaded with 300 nmol of $CaCl_2$. Arsenazo III was used as the calcium indicator. Local anesthetics were administered just prior to initiation of Ca^{2+} loading. At the completion of loading, 40 μl of concentrated Ca-EGTA solution was added so that free $[Ca]_0$ was fixed at either 10^{-4} or 10^{-8} M. Aliquots (0.1 ml) were then withdrawn, rapidly filtered (see "Experimental Procedures"), and filtrates were counted for ^{45}Ca. The Ca^{2+} efflux rate of the control was 3.35 ± 0.08 (4) at 10^{-8} M and 1.39 ± 0.19 (4) at 10^{-4} M. The net Ca^{2+} release rate of the control, measured with arsenazo III, was 2.43 ± 0.08 (5). Such rates are expressed as μmol of Ca^{2+}/min·mg of protein and are means ± S.D. for the number of determinations shown in parentheses.

	Rate of Ca^{2+} release (% of control)	Rate of Ca^{2+} efflux (% of control)	
		$pCa_0 = 8$	$pCa_0 = 4$
Tertiary amines			
SKF 525-A, 40 μM	48.9	70.8	102.8
SKF 525-A, 60 μM	35.7	51.0	94.7
Procaine, 10 mM	87.0	86.7	91.2
Neutral			
Benzocaine, 4 mM	53.3	75.0	56.6
Benzocaine, 6 mM	33.9	64.3	20.3
Quaternary amines			
QX 572, 0.2 mM	51.1	59.1	96.5
QX 572, 0.25 mM	36.4	48.1	96.3
QX 314, 0.2 mM	102.6	ND[a]	91.3
QX 314, 0.25 mM	95.2	98.2	ND

[a] ND, not determined.

Effect of Local Anesthetics on Ca^{2+} Efflux at Low and High Free $[Ca]_0$—We have previously found (43) that Ca^{2+} efflux rates display two maxima as a function of free $[Ca]_0$ during efflux, one around 10^{-8} to 10^{-7} M, associated with spontaneous Ca^{2+} release, the other at 10^{-4} M is smaller. Since the mechanism underlying Ca^{2+} release may be different at low and high $[Ca]_0$ (*i.e.* spontaneous *versus* a possible Ca^{2+}-induced Ca^{2+} release; see Ref. 8), the action of local anesthetics on Ca^{2+} efflux at two different $[Ca]_0$ was analyzed.

As seen in Table IV, at pCa_0 8, SKF 525-A (40–60 μM), QX 572 (0.2–0.25 mM), and benzocaine (4–6 mM) inhibited Ca^{2+} efflux when administered just prior to Ca^{2+} loading. Procaine (10 mM) and QX 314 (0.25 mM) were largely ineffective. These results are in good agreement with those obtained by other assays described earlier. In contrast, at pCa_0 4, only benzocaine (4–6 mM) appreciably inhibited Ca^{2+} efflux (Table IV).

DISCUSSION

This study provides the first detailed analysis of the effect of local anesthetics on spontaneous Ca^{2+} release, and shows that local anesthetics inhibit this form of Ca^{2+} release. The inhibition is not instantaneous but time-dependent and varies widely with respect to the specific local anesthetic. This study suggests the existence of a Ca^{2+} pathway (channel) which is blocked by anesthetics from the inner face of the SR membrane.

Model of Action of Local Anesthetics—The time dependence of inhibition of Ca^{2+} release (Figs. 3, 4 and 6) or Ca^{2+} efflux (Fig. 7) by local anesthetic might be explained either by slow binding of local anesthetic to an inhibitory site, or slow crossing of the membrane to reach the inhibitory site, or a combination of both. Previous studies (45) suggested an intrinsically rapid local anesthetic-receptor interaction. It seems unlikely that the time scale of binding would differ drastically between fairly similar compounds *i.e.* lidocaine and tetracaine reached equilibrium within minutes while their respective

analogs, QX 314 and procaine, did not reach equilibrium even after 4 h of preincubation. It seems more likely that the increased effectiveness of local anesthetics with incubation is due to time required for the anesthetic to permeate inside the SR vesicles. In this regard, we find that QX 314, which is poorly permeant, is effective only after prolonged preincubation, while the more permeant QX 572 (45) is rapidly effective.

Our model is that local anesthetics have to cross the SR membrane before reacting with an internal binding site(s). The following four consecutive steps might be involved in inhibition of Ca^{2+} release by local anesthetics: 1) insertion into the external leaflet of the membrane, 2) distribution (flip-flop) between the two halves of the bilayer, 3) dissociation from the membrane into the aqueous internal milieu, and 4) association with the putative binding site. Different local anesthetics appear to be rate-limited in their onset of action by different steps.

The hydrophobic partition coefficient (F) would reflect the ability of the anesthetic to insert into the membrane (step 1). In order for flip-flop to occur, the anesthetic must be in the uncharged form (45, 50, 51). Thus, flip-flop (step 2) is a function of both F and the percentage of free base form at a specific pH. Step 3 would not be necessary if the anesthetic can reach the binding site while inserted into the membrane. However, the direct correlation between halftime to reach equilibrium and hydrophobicity (Fig. 8*a*) suggests that dissociation from the membrane is an obligatory and rate-limiting step for most of the local anesthetics studied (Table III). More hydrophobic compounds such as SKF 525-A, etidocaine, and bupivacaine would be expected to dissociate more slowly from the membrane into the internal aqueous environment and this would be expected to retard binding to the inhibitory site when access is only via a hydrophilic pathway.

Procaine, procainamide, and QX 314 were not included in Fig. 8*a* because they behave differently, taking much longer time (hours) to exert an effect. For these three anesthetics, which are poorly permeant and 98 to 100% in a charged form, it would seem that step 1 and/or 2 of the model is rate-limiting. The finding that prilocaine, less hydrophobic but with a higher percentage of free base form than procaine (Table I), attains equilibrium after a few minutes (Table III), whereas procaine does not reach equilibrium even after 4 h of preincubation, suggests that step 2 is likely to be rate-limiting for procaine. Since at pH 7.0, prilocaine contains 10-fold more of the free base form than procaine (Table I), it is possible that flip-flop is not rate-limiting for prilocaine but is for procaine. The same explanation can be advanced to interpret the increasing inhibition of Ca^{2+} efflux by procaine above pH 7.0 (Fig. 9*a*).

Comparison of the potency of different local anesthetics at equilibrium should reflect their association with a binding site (step 4) and express selectivity of interaction. The IC_{50} (Table III) spans about three orders of magnitude. We find a direct relationship between M_r (see Table I) and potency at equilibrium (correlation coefficient 0.71). This conforms with a suggestion by Courtney (60) that the dissociation rate is inversely related to the size of local anesthetics. There is poor correlation between lipid solubility and potency (Fig. 8*b*). However, when QX 572 (way out of line in Fig. 8*b*) is not considered, the correlation improves (correlation coefficient −0.77). This implies that hydrophobic interaction may be an integral component of the binding site, as recently reported by Wang *et al.* (61) for the Na^+ channel of squid axon membranes.

A number of observations, *e.g.* the action of local anesthetics below pH 7.0 (Fig. 9), is best explained by pH-dependent changes of the SR binding site (step 4). These findings would suggest that the binding affinity for charged local anesthetics may be higher at low pH. The possibility of higher nonspecific membrane permeability at acidic pH is ruled out by the ineffectiveness of QX 314. The pH independence of benzocaine action is compatible with a different (although still internal) site of action for neutral local anesthetics (62–64). Alternatively, both neutral and charged local anesthetics may bind to the same site (45, 51, 60) with protonation increasing affinity only for charged anesthetic molecules (51).

Evidence in favor of lipid *versus* protein as sites of action of local anesthetics on membranes has been presented (70). The mode of action described here indicates that the SR binding site for local anesthetics is protein in nature, since evidence for an inhibitory site on the inner face of the SR membrane is provided.

Selectivity of Action on a Ca^{2+} Channel—It appears unlikely from the studies presented, that local anesthetics inhibit spontaneous Ca^{2+} release by blocking monovalent ion permeability pathways in SR. The lack of effect of the protonophore FCCP implies that the proton gradient which transiently develops during Ca^{2+} uptake (65) is not relevant to spontaneous Ca^{2+} release or that it is dissipated before Ca^{2+} release takes place. This finding is in agreement with reports by Louis *et al.* (66) and Shoshan *et al.* (67) that protonophores do not induce Ca^{2+} release from isolated SR. Additionally, should action of local anesthetics have involved inhibition of proton or monovalent cation permeability, spontaneous Ca^{2+} release should have been restored by FCCP or gramicidin D, respectively. This was not the case. On the other hand, when Ca^{2+} permeability was specifically increased with A23187, a Ca^{2+} ionophore, inhibition of Ca^{2+} release by local anesthetics was circumvented.

Experiments performed using an anion permeability blocker (DIDS) and K^+ channel blockers (bis-Q10 and bis-Q12) indicate that inhibition of Ca^{2+} release by local anesthetics cannot be attributed to a primary effect on these permeabilities. In fact, DIDS enhances Ca^{2+} release because the phosphate-facilitated Ca^{2+} re-uptake is blocked. The results obtained with bis-Q10 and bis-Q12 are less than conclusive since we could not assess whether the bis-quaternary ammonium blockers were able to get to their internal blocking site or if a membrane potential (negative inside) favorable to such a block (58) existed. However, Miller[6] found that K^+ conductance in a planar phospholipid bilayer containing K^+ channels from SR (52) was not inhibited by 0.5 mM SKF 525-A and only partially inhibited by 2 mM QX 572 *i.e.* concentrations which are 10-fold higher than those required to inhibit spontaneous Ca^{2+} release from SR.

Taken together, our results are compatible with the view that local anesthetics interact directly (7, 16, 21–23, 26) with a Ca^{2+} pathway *i.e.* the Ca^{2+} channel or its gates, rather than with other ion pathways.

Spontaneous Ca^{2+} Release Differs from Other Types of Ca^{2+} Release—It has been reported that local anesthetics inhibit, *without preincubation*, Ca^{2+}-induced Ca^{2+} release (3, 8, 10, 39), caffeine-induced Ca^{2+} release (3, 9, 10, 19), and ADP-induced Ca^{2+} release *i.e.* pump reversal (31) but not depolarization-induced Ca^{2+} release (13, 41, 42). Conceivably, there could be as many Ca^{2+} pathways as Ca^{2+} release mechanisms. Spontaneous Ca^{2+} release could have its own triggering mechanism and efflux pathway,[2] as well as a distinct inhibitory

[6] C. Miller, personal communication.

binding site for local anesthetics. To our knowledge, this is the first report suggesting the presence of a binding site for anesthetics on the internal side of the SR membrane. Asymmetric binding sites for anesthetics have also been described for channel proteins of other membrane systems (45, 49, 60, 64, 69).

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