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Effects of local anesthetics on the passive permeability of sarcoplasmic reticulum vesicles to Ca^{2+} and Mg^{2+}

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Sarcoplasmic reticulum vesicles are used here as model membrane system to question the hypothesis of enhancement of permeability of cations by anesthetics, particularly that of Ca^{2+} and of Mg^{2+}. The effects of dibucaine (up to 800 μM), tetracaine (up to 2 mM), lidocaine (up to 10 mM) and procaine (up to 10 mM) on the permeability of these membranes to Ca^{2+} and Mg^{2+} have been measured. We have used an experimental approach based on the light scattering method (Kometani, T. and Kasai, M. (1978) J. Membrane Biol. 41, 295–308). It has been found that all the local anesthetics cited above markedly increase the permeability of sarcoplasmic reticulum vesicles to Mg^{2+} and, in the concentration range tested herein, only dibucaine and tetracaine increase the permeability to Ca^{2+}. The kinetic analysis of the time dependence of the light-scattering data after the osmotic shock shows that, in the absence of local anesthetics, the Mg^{2+} influx can be described as proceeding through a unique type of channel. However, Ca^{2+} influx appears to involve two channel of different kinetic properties. Because the relative fraction of both types of Ca^{2+} channel is similar to the average ratio between light and heavy vesicles in unfractionated sarcoplasmic reticulum, we suggest that each type of channel can be preferentially located in one of these fractions. The determined rate constants for Ca^{2+} permeability through both types of channel are 0.77 ± 0.08 min^{-1} (fast channels) and 0.025 ± 0.005 min^{-1} (slow channels) and that for Mg^{2+} is 0.08 ± 0.02 min^{-1}. These results agree with data obtained by other groups using different experimental approaches. Dibucaine and tetracaine significantly alter the rate of Mg^{2+} and Ca^{2+} influx through the slow channels. In addition,these two local anesthetics also produce the effect that the Mg^{2+} influx cannot be described with only one exponential process, thus suggesting a differential effect on vesicles of different density. The increase of Ca^{2+} and Mg^{2+} permeability by dibucaine and by tetracaine is found at concentrations of these drugs that do not produce a noticeable inhibition of the (Ca^{2+} + Mg^{2+})-ATPase activity of sarcoplasmic reticulum vesicles.

Introduction

Although a large research effort has been done, anesthesia remains to be satisfactorily explained at a molecular level (see, for example, Refs 1 and 2 for recent reviews on this topic) Two extreme hypothesis have been formulated (1) that

Abbreviations EDTA, ethylenediaminetetraacetic acid, EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, Tes, 2((2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino) ethanesulphonic acid

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anesthetics exert their actions via direct perturbation of specific membrane proteins upon binding to them [3,4] or (ii) that anesthesia results from functional perturbations of membranes as a result of alterations of the physical state of the lipid matrix by anesthetics [5,6]

In any case, the nature of the membrane functional perturbations leading to anesthesia also remains obscure Previously, Johnson and coworkers [7,8] suggested that anesthesia could result from a non-specific enhancement of the permeability of excitable membranes to physiologically relevant cations More recently, Bangham et al [9] have proposed that anesthetics act by increasing the functional proton permeability of membranes, particularly those of synaptic vesicles, and thus it has been called the pump-leak hypothesis Later, it was noted that in the presence of ATP the functioning of a H^+ translocating ATPase in synaptic membrane vesicles could compensate for this increase of H^+ passive flux [10] However, Barchfeld and Deamer [10] have shown that the increase of permeability of H^+ induced by general anesthetics through liposome membranes is not markedly different than that induced for K^+ at concentrations close to their pharmacological doses

In this respect, it is worthwhile to recall here that many of the biochemical actions of local anesthetics are expressed in Ca^{2+}-dependent processes [11] Electrophysiological studies have shown that the conductance of nerve membranes to Ca^{2+} is perturbed by procaine and that these perturbations are reverted by Ca^{2+} [12] Recently, we have shown that the $(Ca^{2+} + Mg^{2+})$-ATPase from synaptosomal membranes is inhibited by local anesthetics at pharmacological concentrations of these drugs [13] These observations have led to the hypothesis that anesthetics may depress synaptic transmission as neurotransmitter stores become depleted, due to the non-specific release of neurotransmitter which should follow to a sustained increase of free cytosolic Ca^{2+} [10,13]

It is also known that some local anesthetics inhibits the mitochondrial F_1-ATPase [14,15] and the $(Ca^{2+} + Mg^{2+})$-ATPase from sarcoplasmic reticulum [16–18] It is also well known that in malignant hyperthermia induced by general anesthetics the ability of the sarcoplasmic reticulum to requester Ca^{2+} is drastically impaired [19] On the other hand, the rate of Ca^{2+} fluxes across sarcoplasmic reticulum membranes is similar to those currently found in other membranes of excitable tissues [16] In addition, this membrane is one the membranes better characterized at a biochemical level

For these reasons, we have chosen sarcoplasmic reticulum membrane preparations to check the hypothesis of enhancement of cations permeability by anesthetics For simplicity in handling, we have used local anesthetics as model anesthetics. It has to be noted that sarcoplasmic reticulum membrane likely undergo structural changes on a narrow pH range near 7 0, as reflected by the steep pH dependence of the ATPase activity around this pH [20] Therefore, we have not attempted to check the effects of local anesthetics on H^+ permeability, because these studies imply the establishment of a ΔpH of, at least, 1 unit and it has been shown that these membranes are highly permeable to H^+ [21]

To study the effects of these anesthetics on sarcoplasmic reticulum permeability to physiologically relevant cations we have used an experimental approach that involves the distortion of membrane vesicles by osmotic shock with a hyperosmotic solution, as described in Ref 22 After the osmotic shock, the internal volume of the vesicles decreases sharply due to water outflow and, then, the vesicles slowly recover their spherical shape in a process that closely follows the inflow of the ionic species that have been used to produce the osmotic shock [22]

This procedure offer several advantages with respect to the use of tracer methods First, radioactive tracers of physiologically relevant cations (for example, Mg^{2+}) are not always readily available Second, this approach is less expensive and generates continuous data as a function of time, data that can be mathematically handled for kinetic analysis of the diffusion of ions across the membrane On the other hand, because we have recently found that local anesthetics interact with divalent cation-chelating structures such as EDTA, ATP and EGTA [23], metallochromic indicators of divalent cations have been specifically avoided, because they can generate artifactual results

Materials and Methods

Sarcoplasmic reticulum has been prepared from rabbit skeletal muscle following the protocol of MacLennan [24], except that phenylmethylsulfonyl fluoride (0 1 mM) and β-mercaptoethanol (2 mM) were added to all the preparation buffers The sarcoplasmic reticulum was resuspended in the buffer (50 mM Tes/0 1 M KCl/0 25 M sucrose/2 mM β-mercaptoethanol (pH 7 4), split into small aliquots and frozen at −60 to −70 °C until use, currently within 1 month after their preparation The samples used in this study were thawed only once SDS-polyacrylamide gel electrophoresis of these samples have shown that the protein pattern of sarcoplasmic reticulum membranes is unaffected during storage in these conditions

The protein concentration has been measured following the method of Lowry et al [25], using bovine serum albumin as a standard The (Ca^{2+} + Mg^{2+})-ATPase activity was measured using the coupled enzymatic assay pyruvate kinase-lactate dehydrogenase, as described in Ref 26 The composition of the reaction mixture used in kinetic assays was as follows 0 1 M Tes/0 1 M KCl/0 1 mM $CaCl_2$/5 mM $MgCl_2$/2 5 ATP/0 42 mM phospho*enol*pyruvate/0 2 mM NADH/7 5 IU pyruvate kinase/18 IU lactate dehydrogenase (pH 7 45) The Ca^{2+}-independent ATPase activity was measured in the presence of 3 4 mM EGTA, and it was found to be less than 10% of total uncoupled (Ca^{2+} + Mg^{2+})-ATPase activity The ratio between the (Ca^{2+} + Mg^{2+})-ATPase activity in the presence and absence of A23187 (2 μg/ml) was used as a control parameter to characterize the 'leakiness' of sarcoplasmic reticulum vesicles [27] This ratio has been typically found to range between 2 5 and 4 at 20–22 °C in all the preparations used in this study

Measurements of ionic permeabilities

Ionic permeabilities have been measured following the light scattering method, as applied to sarcoplasmic reticulum by Kometani and Kasai [22] Briefly, the experimental protocol used was as follows. Sarcoplasmic vesicles have been dialyzed to remove buffer components (0 25 M sucrose, 0 1 M KCl and excess Tes) and the composition of their luminal space has been fixed to 10 mM Tes (pH 7 45) In order to avoid an extensive lysis of sarcoplasmic reticulum vesicles, sucrose and KCl were removed in three steps of 1–2 h each, so that the vesicles were dialyzed first against 10 mM Tes/25 mM KCl/100 mM sucrose, then against 10 mM Tes/25 mM KCl and finally against 10 mM Tes The effect of these dialysis treatments on the leakiness of vesicles has been checked using A23187, as indicated above We have found that the dialyzed vesicles retain their ability to generate Ca^{2+} gradients coupled to ATP hydrolysis, as inferred from the fact that their (Ca^{2+} + Mg^{2+})-ATPase activity is stimulated more than 2-fold at 22 °C by A23187

The operational procedure followed in the experiments of osmotic shocks is outlined next 1 vol of dialyzed sarcoplasmic reticulum vesicles preequilibrated at 25 °C is mixed with 1 vol of 10 mM Tes buffer containing the appropriate salt (i e , NaCl, KCl, $CaCl_2$ or $MgCl_2$) at a concentration of 200 mM. The final salt concentration is fixed to 100 mM and the protein concentration diluted to half of its initial value (currently 0 2 mg/ml) We also confirmed that lower salt concentrations (i e , 25, 50 and 75 mM) gave us similar time dependence, but in these latter cases the amplitude of the scattering change was much smaller, therefore increasing the noise to signal ratio

Light scattering intensity at 400–410 nm has been measured at 25 °C as a function of time after mixing using a Hitachi/Perkin Elmer spectrofluorimeter, model 650-40 Because of the light absorption of dibucaine in the wavelength range 400–410 nm, in experiments involving this local anesthetic the light scattering measurements were carried out at 450–460 nm

Due to the low solubility of local anesthetics in water, to reach the higher local anesthetics concentrations used in this study we used concentrated stock ethanolic solutions Separate control experiments have shown that ethanol concentrations below 2% (v/v) do not significant alter the influx rate of the salts used in this study across the sarcoplasmic reticulum membrane Thus, care was taken that the ethanol concentrations added together with the local anesthetic never exceeded this value In addition, we carefully checked the

pH of the solutions after addition of ethanolic local anesthetic, for pH changes due to the protonation of the local anesthetics This effect is larger than approx 0 1–0.2 pH units when local anesthetic concentrations are in the millimolar range Whenever necessary, the pH was readjusted to pH 7 45 ± 0 05

As it will be indicated below, the analysis of the data requires the determination of the limiting value of the scattering intensity at long times (t_∞) after mixing Considering the permeability properties of sarcoplasmic reticulum vesicles to the cations studied herein [22], this value has been measured up to 2–3 h after mixing, on gently stirred samples. Measurements carried out at different times between 2 and 6 h after mixing have shown that there is not any appreciable change of light scattering intensity in this period of time In some cases appropriate ionophores or low detergent concentrations (Triton X-100) have been used to dissipate rapidly the ionic gradient established at $t = 0$ and, thus, accelerate the approach to the limiting scattering intensity Both experimental approaches have given us identical results in our experimental conditions

Analysis of the light scattering data

The time dependent decrease of the light scattering intensity after an osmotic shock is related to the passive diffusion of the salt components of the outer medium to the inner space of vesicles [22] As shown by Kometani and Kasai [22], in the case of sarcoplasmic reticulum vesicles chloride ions are much faster permeant species than are cations Therefore, the decrease of the light scattering intensity after the osmotic shock reflects the equilibration of the cation across the membrane

On the other hand, it has been shown [28] that the following relationship between internal volume, V_i, and scattering intensity, I_{90}, holds for liposomes subjected to osmotic deformations

$$\Delta V_i \alpha (\Delta I_{90})^{3/2} \tag{1}$$

Because water must accompany the cation diffusion within the luminal space of the vesicles in order to maintain isoosmolarity during the recovery of the liposomes' shape [22], for the case of a simple passive diffusion process defined by a diffusion constant (D_{cation}) we can write

$$(V_\infty - V_t) = (V_\infty - V_o)\ e^{-D_{\text{cation}}\, t} \tag{2}$$

where V_∞, V_o and V_t are the limiting volume (volume of the relaxed spherical state), the volume of the vesicles at the beginning of the phase of recovery of spherical shape and the volume at a time t after the osmotic shock, respectively.

Combining Eqns 1 and 2, it can be derived that

$$|I_\infty - I_t|^{3/2} = |I_\infty - I_o|^{3/2} \exp(-D_{\text{cation}}\, t) \tag{3}$$

where I_∞, I_o and I_t are the scattering intensities of the vesicle state defined by V_∞, V_o and V_t, respectively The electroneutrality of the cation passive diffusion is guaranteed in our experimental conditions by the parallel diffusion of chloride anion It follows that it the recovery of spherical shape can be described by a simple diffusion process through a unique type of channel of fixed permeability properties, the plot of (3/2) $\ln |I_\infty - I_t|$ versus t must give a straight line, from where D_{cation} can be estimated

As it will be seen later, in many instances clear and significant deviations from linearity have been observed in these types of semilogarithmic plots of the experimental data In these cases, the data have been satisfactorily fitted to the sum of two exponentials

$$|I_\infty - I_t|^{3/2} = |I_\infty - I_o|^{3/2} (A\ e^{-D_1 t} + (1-A)\ e^{-D_2 t}) \tag{4}$$

where D_1 and D_2 are the diffusion coefficients of the cation through (a) two distinct pathways or channels in the membrane or (b) two different conformational states of the same channel with different permeability properties, and A is the fraction of cation channels of type 1 or in conformational state 1

In all the cases, the transformed data have been fitted by linear regression analysis to obtain the best values for D_{cation} In the cases where the sum of two exponentials have been used to fit the data, the operating protocol has been the following First, values for D_2 have been obtained by least-

squares linear regression fit of the data at long times The difference between the transformed data and the corresponding Y value of the straight line fitted to the longer times has been adjusted to another straight line, from where D has been obtained The value of A has been obtained from the ordinate intercepts of the fitted straight lines

Chemicals

ATP, phospho*enol*pyruvate, EGTA, dibucaine, procaine, lidocaine, tetracaine, phenylmethylsulfonyl fluoride, β-mercaptoethanol, imidazole and Tes were obtained from Sigma Pyruvate kinase and lactate dehydrogenase were purchased from Boehringer Mannheim All the other chemicals used in this study were obtained from Merck

Results

As indicated in Materials and Methods, we have measured the slow decrease of the scattering intensity after an osmotic shock with a hyperosmolar salt solution This protocol has been previously used with sarcoplasmic reticulum preparations to measure the influx rate of cations under similar experimental conditions [22] However, vesicle-to-vesicle interactions could be significant, as inferred from the comparison of the data obtained by laser scattering measurements with those obtained by electron microscopy [29], and also from the different reactivity of sarcoplasmic reticulum vesicles against β-mercaptoethanol at 0 1 and 1 mg/ml [30] Moreover, these interactions could be altered by the presence of anesthetics Therefore, we further assessed that a putative dissociation of vesicles does not significantly contribute to the observed light scattering change after the osmotic shock in the presence of these drugs in our experimental conditions

To ascertain this point, we carried out control experiments, in which dialyzed vesicles have been diluted with the same volume of the dialysis buffer in the presence of different local anesthetics concentrations Because in these experimental conditions there is not osmotic induced deformation of vesicles, any change of the light scattering intensity should be directly related to a change in the extent of vesicle interactions We have found no significant light scattering changes of sarcoplasmic reticulum vesicle suspensions at total protein concentrations ranging from 0 10 to 0 25 mg/ml in these control experiments, in the presence of the anesthetic concentrations used in this study In addition, we have not found significant deviations from linearity of the plot of light scattering intensity versus sarcoplasmic reticulum concentration, in the concentration range used in osmotic shock experiments (approx 0–0 2 mg/ml) Control experiments have also been carried out using vesicles preequilibrated with high salt concentrations, i e , 100 mM NaCl, KCl, $MgCl_2$ or $CaCl_2$ Therefore, under our experimental conditions changes in vesicle to vesicle interactions are unlikely to contribute significantly to the measured time-dependent decrease of the light scattering intensity after an osmotic shock by high salt concentrations

The analysis of the results obtained on osmotic shocks with 100 mM NaCl or KCl presents additional difficulties derived from the fact that the influx rate of these salts across sarcoplasmic reticulum membranes is relatively fast ($t_{1/2}$ = 0 2–0 3 min) [21,22] Due to technical limitations, we can only record the tail of this process, i e , the light scattering change at times longer than 15–20 s (results not shown) Considering the results obtained by Kometani and Kasai [22], this tail amounts to about 20–25% of the total light scattering change, which should be about 45% of the final I_∞ value We have found that this part of the scattering change can be fitted to Eqn 3 with a regression coefficient higher than 0 98 and yielding a $t_{1/2}$ of 1 5 min (KCl) and 1 9 min (NaCl) (Table I) These kinetic parameters are very similar to those reported for K^+ and Na^+ permeability across the type II sarcoplasmic reticulum vesicles [21], which must be present in our unfractionated preparations

Table I also lists the apparent $t_{1/2}$ for permeability to $MgCl_2$ and $CaCl_2$ Since the time dependence of the light scattering data after an osmotic shock with 100 mM $MgCl_2$ can be adequately fitted to Eqn 3, the $t_{1/2}$ value for $MgCl_2$ is directly related to its diffusion coefficient across these membranes However, the value of $t_{1/2}$ for $CaCl_2$ is only apparent because the time dependence of the light scattering data after an osmotic shock with 100 mM $CaCl_2$ is best fitted by Eqn 4

TABLE I

APPARENT HALF-TIMES OF SARCOPLASMIC RETICULUM PERMEABILITY TO CATIONS

Cation	This study (influx) (min)	Selected literature data (min)
K^+	< 0 3 1 5±0 1 [a]	0 17 (influx) [b]
Na^+	< 0 3 1 9±0 2 [a]	0 22 (influx) [b] 1 6–2 0 (efflux) [c]
Mg^{2+}	5–11	(2–6) (efflux) [d]
Ca^{2+}	20–30	20 (efflux) [e] > 15–20 (efflux) [f] 9–10 (efflux) [g]

[a] Obtained by fitting to Eqn 3 the last part of the light-scattering decrease after an osmotic shock with 100 mM NaCl or KCl Therefore, it corresponds to a slow component of Na^+ and K^+ influx across the vesicles used in this study

[b] Determined by the osmotic shock technique [22] Slow and fast components were not analyzed separately

[c] Measured by Millipore filtration [21] slow component

[d] Determined using the metallochromic indicator, antipyrylazo III [31] It varies from approx 2 to 6 min depending on the KCl, sucrose and other salts concentrations of the medium at pH 7 0

[e] Measured by Millipore filtration [16]

[f] Estimated from the combined data of Figs 1 and 2 of Ref 32, assuming that on the average 2/3 of the vesicles are light density sarcoplasmic reticulum membranes Experimental approach from measurements Millipore filtration

[g] Measured by Millipore filtration [33]

This implies two different pathways for Ca^{2+} diffusion across the membranes used in this study (see Table II). It is to be recalled that heavy and light sarcoplasmic reticulum vesicles have different Ca^{2+} permeability properties [32] Table I also includes the values of the apparent $t_{1/2}$ for passive fluxes of these cations across sarcoplasmic reticulum membranes determined by other investigators

Effects of local anesthetics on $CaCl_2$ permeability

Figs 1 (A) and 2 show the dependence upon dibucaine and tetracaine concentrations of the time course of the decrease of the light scattering intensity of sarcoplasmic reticulum vesicles after an osmotic shock with 100 mM $CaCl_2$ These results show that dibucaine and tetracaine increase the permeability of these membranes to $CaCl_2$ in a dose-dependent manner In addition, these data have been treated as described in Materials and Methods and, accordingly, plotted as (3/2) $\ln(I_t - I_\infty)$ versus t This is illustrated in Fig 1 (B) for dibucaine data The plots given in this figure show that the data do not fit to a simple exponential process (Eqn 3) (see Materials and Methods) They can be adequately fitted by Eqn 4 instead, thus indicating that the diffusion of Ca^{2+} into the luminal space proceeds through two kinetically different pathways

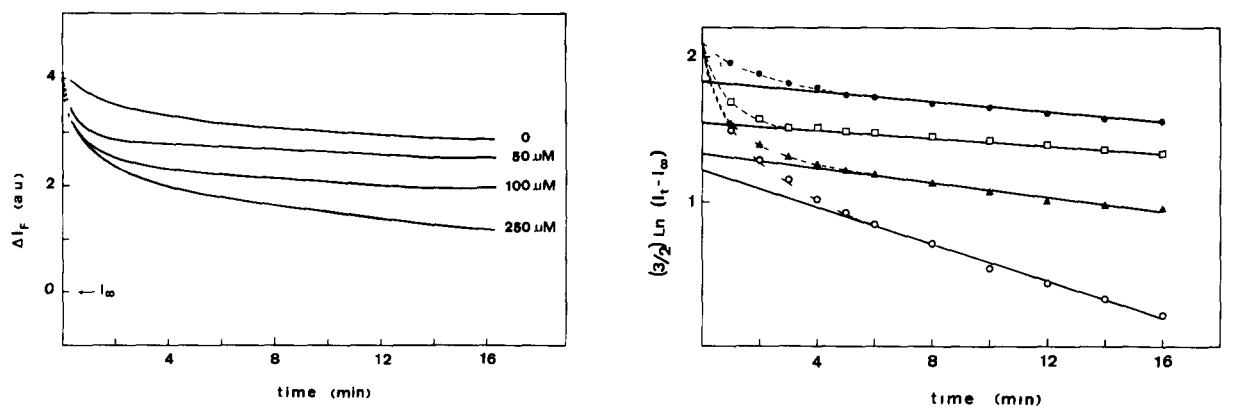

Fig 1 (A) Time dependence of the light scattering intensity of sarcoplasmic reticulum (0 1 mg/ml) after an osmotic shock with 100 mM $CaCl_2$ in the presence of various dibucaine concentrations Total dibucaine concentrations are indicated in the figure The recorder trace obtained in the presence of 800 μM dibucaine lay between those of 100 and 250 μM, showing that 250 μM dibucaine has already saturated the effect of this local anesthetic on the $CaCl_2$ influx rate (B) Semilogarithmic plot of the data shown in (A) The points indicated are only representative data The symbols correspond to the following concentrations (μM) of dibucaine ●, 0, □, 50, ▲, 100, ○, 250

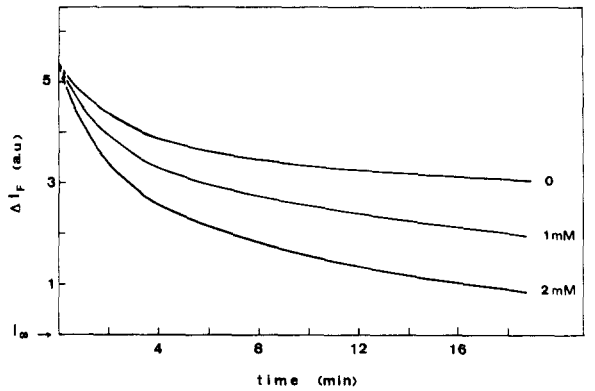

Fig 2 Time dependence of the light scattering intensity of sarcoplasmic reticulum (0 1 mg/ml) after an osmotic shock with 100 mm $CaCl_2$ in the presence of several tetracaine concentrations Total tetracaine concentrations are indicated in the figure

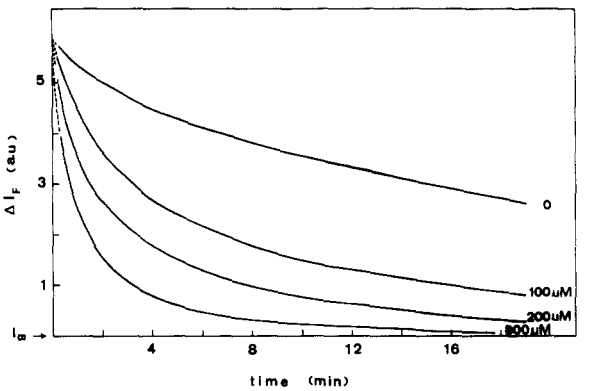

Fig 3 Time dependence of the light scattering intensity of sarcoplasmic reticulum vesicles (0 1 mg/ml) after an osmotic shock with 100 mm $MgCl_2$ in the presence of various dibucaine concentrations Total dibucaine concentrations are indicated in the figure

The results given in Figs 1 (A) and 2 are the results obtained in a typical experimental series with one preparation These results have been confirmed with at least three different preparations In order to check carefully the reliability of the effects of local anesthetics observed, as well as to control any possible alteration of the sarcoplasmic reticulum membrane by storage on ice during a few hours (the time required to complete a experimental series), we repeated the control experiments three times, as follows one at the beginning, another in the middle and the last at the end of each experimental series In general, we noticed that the three control experiments overlap within the experimental noise of the recorded signal It should be noticed, however, that we observed a relatively large variation between control experiments carried out with different preparations This is illustrated in the Table I, in which the limiting values obtained for $t_{1/2}$ have been listed

Lidocaine (up to 10 mM) and procaine (up to 10 mM) do not significantly alter the time course of the dependence of the light scattering intensity after an osmotic shock with 10 mM $CaCl_2$

Effects of local anesthetics on $MgCl_2$ permeability

Fig 3 reproduces the recording traces of the light scattering intensity decrease after an osmotic shock with 100 mM $MgCl_2$ in the presence of several concentrations of dibucaine The data given in this figure have been replotted as (3/2) $\ln(I_t - I_\infty)$ versus t (not shown) These plots show clear deviations from linearity in many instances Thus, for these cases a simple exponential equation (Eqn 3) cannot account for the effects of these anesthetics on the permeability process These data can be fitted to Eqn 4 instead, and the relevant kinetic parameters obtained have been listed in Table II Tetracaine (1 and 2 mM), procaine (10 mM) and lidocaine (10 mM) also increase the passive permeability to $MgCl_2$ (see Table II) (results not shown) In these latter cases, however, the data can be satisfactorily fitted to a simple exponential equation These results show that all the local anesthetics tested herein produce a large increase of the passive permeability to $MgCl_2$, an increase which is dependent on total local anesthetic concentration

As indicated above with respect to permeability to $CaCl_2$, the recording trace obtained after an osmotic shock with 100 mM $MgCl_2$ overlaps within the experimental errors in duplicate experiments carried out with the same preparation However, relatively large variations have been observed for different preparations (see the values of $t_{1/2}$ listed in Table I) For these reasons, the results given in Fig. 3 are those obtained in typical experimental series with the same preparation These effects of local anesthetics on $MgCl_2$ influx across sarcoplasmic reticulum membranes have been confirmed with three different preparations

TABLE II

EFFECTS OF LOCAL ANESTHETICS ON CATIONS PERMEABILITY TO $MgCl_2$ AND $CaCl_2$

Values are averaged from the data obtained in duplicate or triplicate experiments D_1, D_2 and A have been estimated by fitting the time dependence of the light scattering intensity after the osmotic shock to Eqn 4 The correlation coefficient of these fits was always better than 0 98 and the standard deviation ranged from 0 04 to 0 3

Salt used to produce the osmotic shock	Local anesthetic (concentration)	D_1 (min^{-1})	$t^1_{1/2}$ (min)	D_2 (min^{-1})	$t^2_{1/2}$ (min)	A
100 mM $MgCl_2$	None	0 08 ± 0 02	9 ± 2	–	–	–
	Dibucaine (100 μM)	0 52 ± 0 05	1 3	0 11 ± 0 01	6 2	0 34
	Dibucaine (200 μM)	0 46 ± 0 05	1 5	0 16 ± 0 01	4 3	0 34
	Dibucaine (800 μM)	0 69 ± 0 06	1 0	0 29 ± 0 01	2 4	0 34
	Tetracaine (1 mM)	0 49 ± 0 05	1 4	0 32 ± 0 02	2 2	0 46
	Tetracaine (2 mM)	1 22 ± 0 08	0 57	0 37 ± 0 02	1 9	0 56
	Lidocaine (10 mM)	0 39 ± 0 03	1 8	–	–	–
	Procaine (10 mM)	0 26 ± 0 02	2 6	–	–	–
100 mM $CaCl_2$	None	0 77 ± 0 07	0 9	0 025 ± 0 005	28	(0 23 ± 0 03)
	Tetracaine (1 mM)	0 63 ± 0 06	1 1	0 043 ± 0 005	16	0 28
	Tetracaine (2 mM)	0 79 ± 0 08	0 9	0 08 ± 0 01	8 7	0 34
	None	0 77 ± 0 07	0 9	0 025 ± 0 005	28	0 20
	Dibucaine (50 μM)	0 80 ± 0 08	0 9	0 021 ± 0 003	33	0 30
	Dibucaine (100 μM)	0 85 ± 0 05	0 82	0 025 ± 0 003	28	0 40
	Dibucaine (250 μM)	0 78 ± 0 05	0 9	0 065 ± 0 006	10 7	0 41

Effects of local anesthetics on sucrose permeability

Although at concentrations higher than those used in this study, some local anesthetics (such as tetracaine) have been shown to produce micellization of liposomes [34] Due to this reason, to establish the integrity of the membrane structure, we have measured the effects of the local anesthetics at the concentrations used herein on the permeability of sarcoplasmic reticulum membranes to sucrose

The results obtained (not shown) have demonstrated that none of the local anesthetics used decrease markedly the half-time of the light-scattering reequilibration after an osmotic shock with 100 mM sucrose The value that we obtained in the current work for the $t_{1/2}$ of sucrose influx is larger than 120 min In addition, the absolute light scattering intensity of sarcoplasmic reticulum vesicles is not significantly altered by the local anesthetics concentrations used in this study (results not shown)

Therefore, these results strongly suggest that the local anesthetics used in this study do not produce a significant micellization of these membranes under our experimental conditions

Discussion

The results presented in this paper show that local anesthetics have pronounced effects on Mg^{2+} and Ca^{2+} permeability of the sarcoplasmic reticulum Table II summarizes the kinetic parameters obtained from the best fit of the experimental data to Eqns 3 and 4 by least-squares regression analysis

It has been claimed [35] that aggregation of vesicles induced by high concentrations of $CaCl_2$ can lead to erroneous estimation of the permeability of sarcoplasmic reticulum membranes to Ca^{2+} by the osmotic shock technique As indicated in Results, this is unlikely to contribute significantly to the I_{90} changes measured in our experimental conditions Moreover, the $t_{1/2}$ values determined herein for Ca^{2+} and Mg^{2+} influx agree satisfactorily with available literature data reported for Ca^{2+} efflux using the Millipore filtration technique [16,32] and for Mg^{2+} efflux using antipirilazo III [31] However, other investigators have published data for Ca^{2+} efflux of fractionated vesicles that suggest a 5–10-fold higher sarcoplasmic reticulum permeability, using the Millipore

filtration technique as well [33] Sulphydryl protecting reagents, such as β-mercaptoethanol or dithiothreitol, were not present in these studies and it is now known that ageing processes due to the oxidation of sulphydryl by oxygen largely increase the sarcoplasmic reticulum passive permeability to Ca^{2+} [27,36,37] We have also found that, unless oxidation of protein cysteines is prevented, the permeability of sarcoplasmic reticulum vesicles to Ca^{2+} and Mg^{2+} is increased several-fold by ageing processes that develop in a few hours on ice-storage, the time required to prepare them for these experiments, either by dialysis or by preincubation of vesicles with Ca^{2+} (results not shown)

It appears that our results give support to a hypothesis linking anesthesia to altered divalent cations permeability across excitable membranes The results presented in this paper also show that the increase of permeability to divalent cations induced by local anesthetics is specific and it is not related to breakdown of the bilayer structure, because the permeability of sarcoplasmic reticulum vesicles to sucrose is little affected in these experimental conditions In this respect, it should be recalled that extensive NMR studies have shown that the local anesthetics studied herein sit closer to the lipid/water interface region than to the center of the bilayer structure, penetrating through the glycerol backbone and slightly into the fatty acid core of the lipid bilayer [38,39]

Fluidity is also known to play a major role in the modulation of the permeability of ions across biological membranes However, the lipids of the sarcoplasmic reticulum membranes are in the fluid state at 25 °C [40] and, thus, these drugs are not expected to induce important fluidity changes in this membrane We have confirmed this point using diphenylhexatriene polarization measurements (Escudero, B and Gutiérrez-Merino, C, unpublished results) On the other hand, De Boland et al [16] have shown that the permeability of liposomes made of lipids of sarcoplasmic reticulum to Ca^{2+} is 4–5 orders of magnitude lower than that of native vesicles or that of ATPase reconstituted vesicles Dibucaine and tetracaine only increase the Ca^{2+} permeability of liposomes of endogenous lipids 3–4-fold Therefore, it is likely that the effects of local anesthetics on the permeability of sarcoplasmic reticulum to Ca^{2+} and Mg^{2+} reported herein derive from alterations of lipid-protein interactions that contribute to determine the kinetic characteristics of these channels

The data presented in Figs 1–3 and the parameters listed in Table II show that the time dependence of light scattering after an osmotic shock cannot be satisfactorily fitted to only one exponential process in all the cases Therefore, Ca^{2+} or Mg^{2+} influx can be better described in a wide set of experimental conditions as proceeding through two types of channels of different kinetic characteristics The possibility exists that each type of channel is preferentially located in one of the sarcoplasmic reticulum membrane regions, namely cisternae or longitudinal parts Different permeability to cations, Ca^{2+} among them, of both fractions has been already reported [32,41] Moreover, the rate of Ca^{2+} influx across the fast permeating channels derived from the analysis presented here is about the same to that reported for the Ca^{2+} gated channels of the heavy fraction of sarcoplasmic reticulum vesicles [21,32] The estimated weighing factor of the fast permeating channels shows values similar to the weight ratios of the two types of vesicles in unfractionated sarcoplasmic reticulum preparations [42] The experimental data presented in Table II also show that the effects of some of the local anesthetics studied here (such as dibucaine and tetracaine) are clearly different in the two types of channels

It is evident from the comparison of the data given in Fig 4 with those presented in Table II that the concentrations of these local anesthetics that produce a significant increase in the vesicle permeability to $MgCl_2$ and to $CaCl_2$ differ from those required to inhibit the $(Ca^{2+} + Mg^{2+})$-ATPase activity of these preparations A closer observation of these data indicates a complex inhibition pattern, which shows a phase of insensitivity to these drugs at low anesthetic concentrations However, it is to be noted that in native vesicles the $(Ca^{2+} + Mg^{2+})$-ATPase is largely inhibited by the high free Ca^{2+} concentrations in their luminal space which is achieved during active transport [43] Therefore, an increase in Ca^{2+} permeability by dibucaine should also tend to decrease the internal free Ca^{2+} concentration, which in turn will increase the steady-state $(Ca^{2+}$

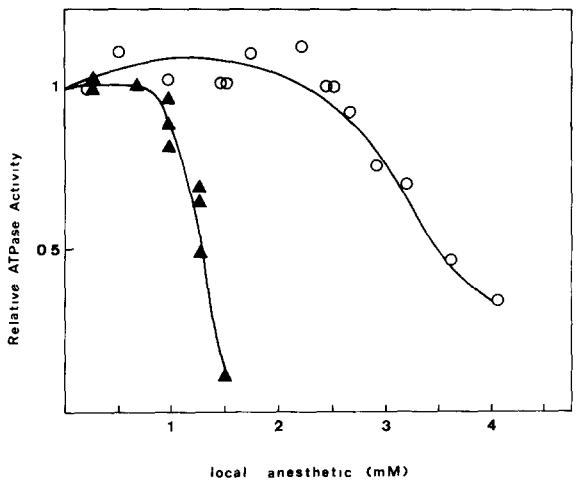

Fig 4 Dependence of the $(Ca^{2+} + Mg^{2+})$-ATPase activity of native sarcoplasmic reticulum vesicles upon the total concentration of dibucaine (▲) and of tetracaine (○) The measurements have been carried out at 25 °C and at pH 7 45 in 0 1 M Tes buffer Other conditions as stated in Materials and Methods

$+ Mg^{2+}$)-ATPase activity in such a way that both effects (i.e, partial inhibition by the anesthetic and activation of ATPase) can cancel each other at the lower anesthetic concentrations

Interestingly, all the local anesthetics tested share the ability to produce a marked increase of the permeability of the sarcoplasmic reticulum membranes to Mg^{2+} It might be worthwhile recalling here that an early and long sustained observation is that raising the extracellular Mg^{2+} concentration decreases cellular excitability in mammals [44] This has been rationalized in terms of Mg^{2+} competition with cytosolic Ca^{2+}-binding sites An increase of Mg^{2+} permeability by anesthetics, such as that reported here, should potentiate these types of effect

Finally, we wish to note that the concentrations needed to elicit a significant increase of passive divalent cations permeability in sarcoplasmic reticulum are between 20 and 50-fold larger than the concentrations needed to reduce the amplitude of the A_α action potentials in sciatic nerve by 50% [4] Therefore, the physiological implications of the findings reported herein are unclear at present, and suggest that the sarcoplasmic reticulum is not the primary site of action of these drugs Nevertheless, when attempting to rationalize the different sensitivity of synaptosomal and sarcoplasmic reticulum $(Ca^{2+} + Mg^{2+})$-ATPase to local anesthetics, it should be recalled that a negative membrane potential can promote a significant accumulation of positively charged lipophilic species within membrane vesicles [45,46]. This accumulation can produce a 10–100-fold increase of lipophilic drugs in nerve membranes that maintain a large steady membrane potential, whereas it will be negligible in sarcoplasmic reticulum vesicles, because these membranes do not raise a large membrane potential during active Ca^{2+} transport [47,48], nor in the experimental conditions used in this study.

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