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ASPECTS OF THE MECHANISM OF ACTION OF LOCAL ANESTHETICS ON THE SARCOPLASMIC RETICULUM OF SKELETAL MUSCLE

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SUMMARY

1. The effect was studied of local anesthetics (tetracaine, dibucaine, procaine and xylocaine) on the forward and the backward reactions of the calcium pump of skeletal muscle sarcoplasmic reticulum.

2. The inhibition of the rate of calcium uptake, the rate of calcium-dependent ATP splitting and the rate of calcium-dependent ATP-ADP phosphate exchange by sarcoplasmic reticulum in the presence of the above drugs is at least partially due to the inhibition of the phosphoprotein formation from ATP.

3. The rate of the ADP-induced calcium release from sarcoplasmic reticulum and the rate of ATP synthesis driven by the calcium efflux are inhibited on account of a reduction of the phosphoprotein formation by orthophosphate.

4. The phosphorylation of calcium transport ATPase by either ATP or orthophosphate is diminished by the local anesthetics owing to a reduction in the apparent calcium affinity of sarcoplasmic reticulum membranes on the outside and on the inside, respectively.

5 The drug-induced calcium efflux from calcium-preloaded sarcoplasmic reticulum vesicles, a reaction not requiring ADP, is probably not mediated by calcium transport ATPase.

INTRODUCTION

Several investigations have been undertaken on the effects of local anesthetics on sarcoplasmic reticulum function [1–6]. Local anesthetic drugs such as procaine, tetracaine, dibucaine and xylocaine were shown to inhibit the calcium pump of sarcoplasmic reticulum [7–15] since they reduce the rate of ATP-driven calcium uptake and the rate of calcium-dependent ATP splitting [1–4]. Phosphoprotein formation assayed in the presence of 5 mM calcium was not affected by local anesthetics [4]. Furthermore procaine prevented calcium release from calcium-preloaded cardiac and skeletal muscle sarcoplasmic reticulum by caffein [1, 5], as well as the caffein-induced calcium release and calcium-induced calcium release from sarcoplasmic reticulum of Natori fibers [6]. On the other hand, local anesthetic drugs seem to

release calcium from calcium-preloaded sarcoplasmic reticulum at concentrations which inhibit calcium uptake [1, 3, 4].

Balzer et al. have demonstrated that prenylamine, which inhibits calcium uptake and calcium ATPase [16], also affects the reversal of the calcium pump [17–23], since the drug inhibits calcium-driven ATP synthesis [18]. In addition, β-blocking agents with local anesthetic properties inhibit both the rate of calcium uptake and the rate of ADP-dependent calcium efflux [24].

The aim of the present study was to obtain some information about the steps at which local anesthetic drugs might affect the reaction sequence of transport ATPase-mediated calcium translocation through sarcoplasmic reticulum membranes in both directions. Moreover, information was sought as to whether drug-induced calcium release from sarcoplasmic reticulum vesicles might occur via the calcium transport ATPase as well. Some of the results have been briefly reported [25, 26].

MATERIALS AND METHODS

Reagents. Ortho[^{32}P]phosphate was purchased from the Radiochemical Centre Ltd. (Amersham). ATP, ADP, procaine and xylocaine were purchased from Sigma Chemical Co. (Saint Louis). Phosphoenolpyruvate, pyruvate kinase, hexokinase and ADP were purchased from Boehringer, GmbH (Mannheim). Thin-layer chromatography aluminium sheets, polyethyleneimine-cellulose F pre-coated and silica gel 60 F_{254} precoated were obtained from E. Merck (Darmstadt). Ethyleneglycol-bis-(2-aminoethyl-ether)-*N*,*N*′-tetraacetic acid (EGTA) was purchased from Fluka, AG (Buchs). Tetracaine and nupercaine were a generous gift from Ciba-Geigy, GmbH (Basel).

^{32}P-labelling of ATP and ADP: [γ-^{32}P]ATP was prepared according to Glynn and Chappell [27] and [β-^{32}P]ADP was prepared according to Makinose [11].

Preparation of sarcoplasmic reticulum. Sarcoplasmic reticulum vesicles of skeletal muscle were prepared from rabbits according to Hasselbach and Makinose [28]. A solubilised ATPase preparation, prepared by solubilisation of sarcoplasmic reticulum vesicles with deoxycholate, followed by fractionation with ammonium acetate, according to MacLennan [29] was used in some of the experiments.

Analyses. Protein was determined by the method of Daughaday et al. [30] or by the biuret method after dispersion with deoxycholate, using crystalline bovine serum albumin as standard. Calcium uptake and ATPase activity were measured as previously described [31–33]. The basic ATPase activity was assayed in the absence of calcium and in the presence of 0.2–0.5 mM EGTA. The calcium-dependent ATPase was calculated by subtracting the basic ATPase from the total ATPase, which was measured in the presence of calcium. Inorganic phosphate was determined according to Rockstein and Herron [34]. ATP-ADP phosphate exchange was performed and calculated as described in detail by Makinose [11], except that the separation of the nucleotides was carried out by thin-layer chromatography using either polyethyleneimine-cellulose (solvent: 2 M formic acid/0.5 M LiCl) [35] or silica gel (solvent: *n*-propanol/ammonium (33 %)/methanol/water (45 : 30 : 15 : 10, v/v)) [36].

Phosphoprotein formation by sarcoplasmic reticulum from [^{32}P]ATP or ortho[^{32}P]phosphate was carried out according to Makonose [11, 19]. Phosphoenolpyruvate and pyruvate kinase were used when the phosphoprotein formation was

carried out with [^{32}P]ATP in order to remove ADP; since the reaction was stopped within 3–5 s by $HClO_4$, the specific activity of ATP remains more or less unchanged. Calcium release and ATP synthesis were measured according to Makinose and Hasselbach [17]. Glucose (100 mM) and hexokinase (40–80 μg/ml) were present in the incubation medium. Ortho[^{32}P]phosphate was separated from glucose 6-[^{32}P]-phosphate by extraction with isobutanol/benzene/molybdate and the glucose 6-phosphate was estimated by paper chromatography [37].

The passive calcium binding by sarcoplasmic reticulum membranes was assayed in the absence of ATP by equilibrium dialysis [42]. 1.5 mg sarcoplasmic reticulum protein was resuspended in 40 mM histidine buffer (pH 7.0), 100 mM KCl (0.5 ml volume) and dialysed against a medium containing 40 mM histidine buffer (pH 7.0), 100 mM KCl and 10^{-5} M $^{45}CaCl_2$ for 24–30 h. The actual measured calcium concentration in the medium assayed by atomic absorption spectrometry [31] was $1.5 \cdot 10^{-5}$–$1.7 \cdot 10^{-5}$ M in the absence or in the presence of 10^{-3} M tetracaine or 10^{-3} M dibucaine and used for the calculations.

RESULTS

Effect of local anesthetics on phosphoprotein formation by sarcoplasmic reticulum from [^{32}P]ATP

The effect of local anesthetics on [^{32}P]phosphate incorporation from ATP

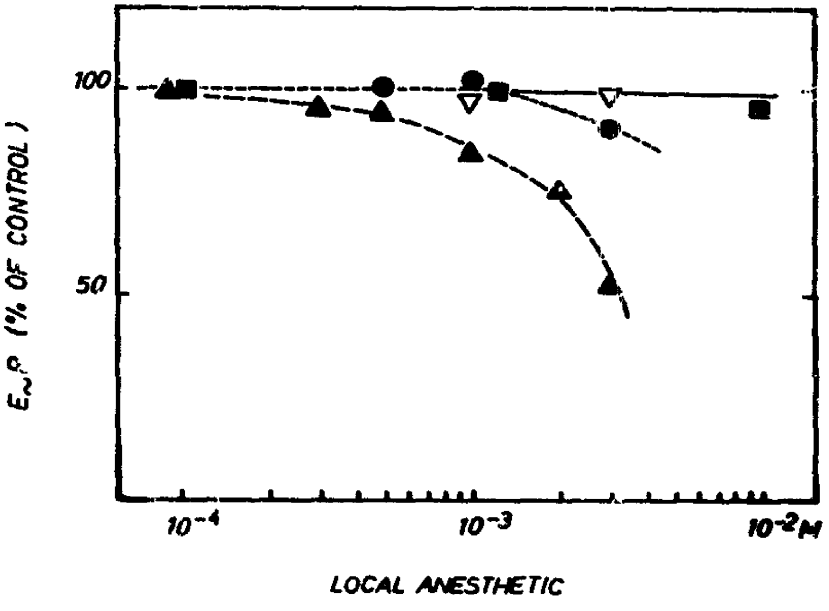


Fig. 1. Effect of various concentrations of tetracaine, dibucaine, procaine and xylocaine on ^{32}P-labelled phosphoprotein formation by sarcoplasmic reticulum from [^{32}P]ATP. Medium: 40 mM histidine buffer (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, 5 mM [^{32}P]ATP, 2 mM phosphoenolpyruvate, 0.02 mg pyruvate kinase/ml, 0.1 mM $CaCl_2$, 0.5 mg sarcoplasmic reticulum protein/ml. T = 23 °C. The reaction was stopped 5 s after addition of the sarcoplasmic reticulum protein by 0.1 M $HClO_4$ containing 16 mM ATP and 10 mM orthophosphate. After five washings with 0.1 M $HClO_4$ the radioactivity of the protein was measured. ●—●, tetracaine; ▲—▲, dibucaine; ■—■, procaine; ▽—▽, xylocaine.

into the ATPase protein of sarcoplasmic reticulum membranes depends on the calcium concentration in the medium. In the presence of 10^{-4} M calcium, there is little effect on the phosphoprotein steady-state level of tetracaine and dibucaine at concentrations of 10^{-3} M and $3 \cdot 10^{-4}$ M, respectively (Fig. 1). However, phosphoprotein levels fall slightly with higher concentrations of tetracaine and more steeply with increasing concentrations of dibucaine. Procaine and xylocaine are without effect (Fig. 1). When the calcium concentration is raised to 1 mM with otherwise identical concentrations to those given in Fig. 1, there is no effect on the phosphoprotein steady-state level even at the highest concentrations of drugs tested. Under these latter conditions the phosphoprotein steady-state levels obtained in the presence of 10^{-3} M dibucaine, $3 \cdot 10^{-3}$ M tetracaine, $3 \cdot 10^{-3}$ M xylocaine and 10^{-2} M procaine, respectively, ranged between 4.33 and 4.66 nmol/mg protein compared to 4.51 nmol/mg protein measured in the absence of drugs.

On the other hand, greatly reduced phosphoprotein steady-state levels are obtained at low ionized calcium concentrations (Fig. 2), demonstrating a shift in the calcium dependence of phosphoprotein formation towards higher calcium concentrations in the presence of the drugs.

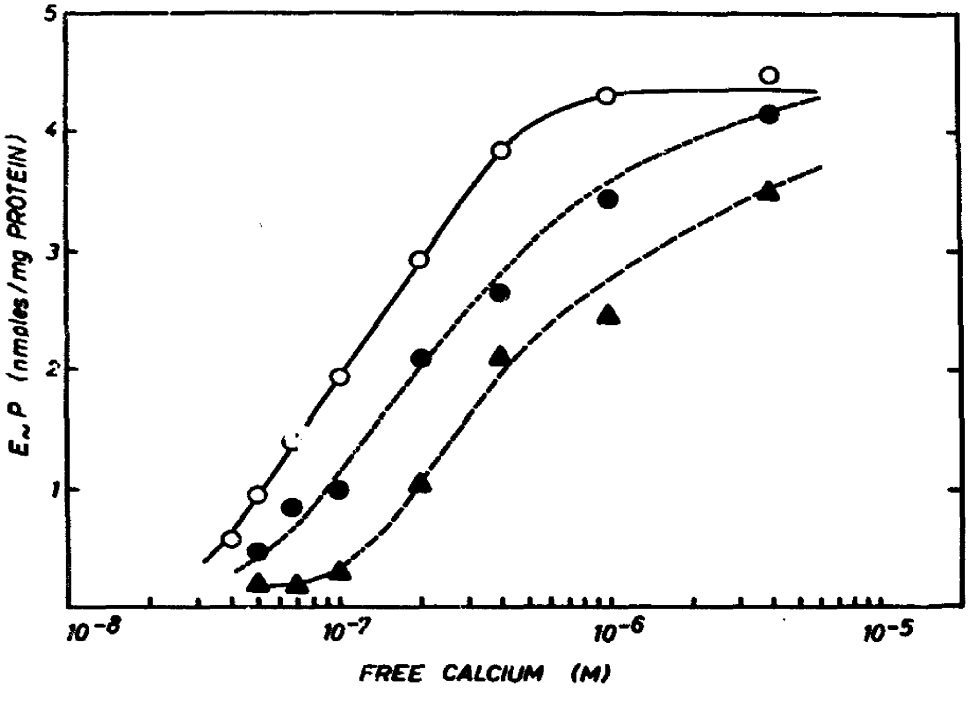


Fig. 2. Effect of tetracaine and dibucaine on ^{32}P-labelled phosphoprotein formation by sarcoplasmic reticulum from [^{32}P]ATP at various pCa. Medium: 60 mM histidine buffer (pH 7.0), 40 mM KCl, 5 mM $MgCl_2$, 5 mM [^{32}P]ATP, 2 mM phosphoenolpyruvate, 0.02 mg pyruvate kinase/ml, 0.5 mM total $CaCl_2$, 0.5–2.5 mM EGTA, 0.5 mg sarcoplasmic reticulum protein/ml. $T = 23$ °C. A value of $2 \cdot 10^{-7}$ M was used as apparent dissociation constant of the Ca^{2+}-EGTA complex in order to calculate the ionized calcium concentration [48]. ○—○, control; ●—●, 10^{-3} M tetracaine; ▲—▲, 10^{-3} M dibucaine.

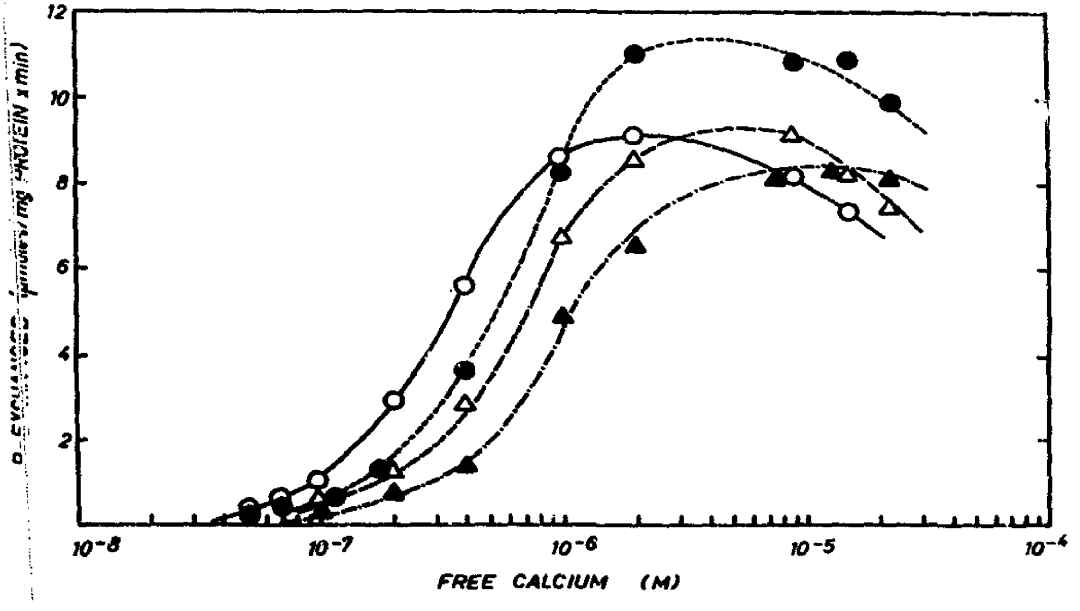
Effect of local anesthetics on ATP-ADP phosphate exchange by sarcoplasmic reticulum

The rate of ATP-ADP phosphate exchange is markedly reduced at low ionized calcium concentrations by tetracaine and dibucaine (Fig. 3). Both drugs shift the calcium dependence of the phosphate exchange reaction towards higher calcium concentrations as observed with phosphoprotein formation (Fig. 2), i.e. the apparent calcium affinity of sarcoplasmic reticulum membranes is reduced in the presence of the drugs. At higher free calcium concentrations there is an increase in the rate of ATP-ADP phosphate exchange which may partially result from an alteration in substrate inhibition.

The greatest increase in the rate of ATP-ADP phosphate exchange occurs in the presence of 10^{-3} M tetracaine and 10^{-3} M dibucaine and is observed at various ADP-ATP ratios ranging from 0.1 to 0.8 (Fig. 4); procaine and xylocaine are without effect under these conditions when the drug concentrations are increased from 10^{-4} to 10^{-2} M and $3 \cdot 10^{-3}$ M, respectively.

The effect of tetracaine and dibucaine on the ATP-ADP phosphate exchange is not due to an alteration in ADP sensitivity of the phosphoprotein formed, since the phosphoprotein steady-state level as well as the rate of the calcium-dependent ATP splitting are reduced to the same extent by ADP either in the presence or in the absence of the drugs (Table I).

A similar increase in the rate of ATP-ADP phosphate exchange by tetracaine and dibucaine under conditions described in Fig. 4 is also observed with a purified



F g. 3. Effect of tetracaine and dibucaine on ATP-ADP phosphate exchange by sarcoplasmic reticulum at various pCa. Medium: 60 mM histidine buffer (pH 7.0), 40 mM KCl, 7 mM $MgCl_2$, 5 mM ATP, 2 nM [^{32}P]ADP, 0.5 mM total $CaCl_2$, 0.5–2.5 mM EGTA (or 0.2 mM total $CaCl_2$ and 0.2–1.25 mM EGTA), 0.03 mg sarcoplasmic reticulum protein/ml. T = 25 °C. Calculation of the ionized calcium concentration as in Fig. 2. ○—○, control; ●—●, 10^{-3} M tetracaine; △—△, $3 \cdot 10^{-4}$ M dibucaine; ▲—▲, 10^{-3} M dibucaine.

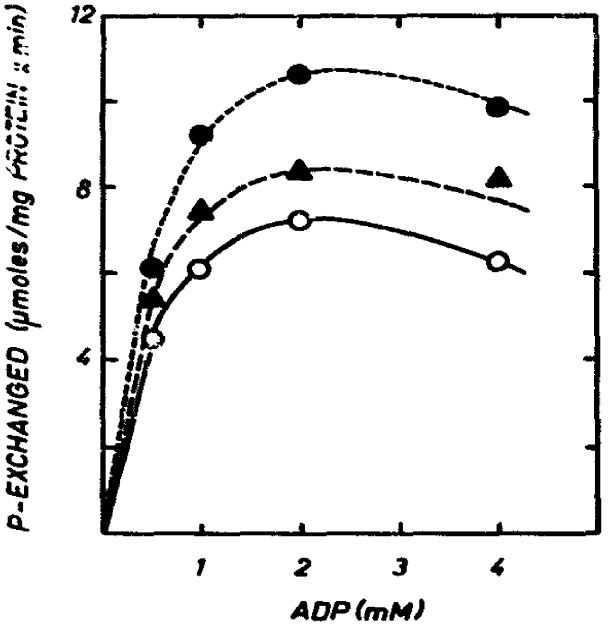


Fig. 4. Effect of tetracaine and dibucaine on ATP-ADP phosphate exchange by sarcoplasmic reticulum at various ADP concentrations. Medium: 40 mM histidine buffer (pH 7.0), 100 mM KCl, 5 mM ATP, 0.5–4.0 mM [^{32}P]ADP, 0.1 mM $CaCl_2$, 0.03 mg sarcoplasmic reticulum protein/ml; $MgCl_2$ concentration was the sum of the nucleotide concentration. T = 25 °C. ○—○, control; ●—●, 10^{-3} M tetracaine; ▲—▲, 10^{-3} M dibucaine.

TABLE I

EFFECT OF ADP ON PHOSPHOPROTEIN FORMATION FROM [^{32}P]ATP AND CALCIUM-DEPENDENT ATPase OF SARCOPLASMIC RETICULUM IN THE PRESENCE OF TETRACAINE AND DIBUCAINE

Phosphoprotein formation was assayed as described in Fig. 1 either in the presence of 5 mM [^{32}P]-ATP plus 2 mM ADP but without phosphoenolpyruvate and pyruvate kinase. The magnesium concentration was the sum of the nucleotide concentration. The Ca^{2+}-ATPase was prepared according to MacLennan [29]. The assay medium contained 40 mM histidine buffer (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 2 mM phosphoenolpyruvate, 0.02 mg pyruvate kinase/ml, 0.5 mM $CaCl_2$, 0.5 mM EGTA, 0.05 mg sarcoplasmic reticulum protein/ml. In the presence of 5 mM ATP plus 2 mM ADP the phosphoenolpyruvate and pyruvate kinase were omitted. Tetracaine and dibucaine concentrations were 10^{-3} M, except in the ATPase assay dibucaine was $3 \cdot 10^{-4}$ M.

	Ca^{2+}-ATPase (μmol P_i/mg protein per min)			E ~ P (nmol/mg protein)		
	5 mM ATP	5 mM ATP + 2 mM ADP	%	5 mM ATP	5 mM ATP + 2 mM ADP	%
Control	5.33	2.00	38	4.38	1.58	36
Tetracaine	3.20	1.17	37	4.08	1.59	39
Dibucaine	2.96	0.95	32	3.85	1.31	34

ATPase preparation [29]. This indicates that the effect of the drugs on the phosphate exchange reaction is independent of calcium-bound to calcium binding proteins [41], since the latter were removed during the preparation of the ATPase [29].

Effect of local anesthetics on calcium uptake, calcium-dependent ATPase and calcium binding of sarcoplasmic reticulum

The effects of various concentrations of tetracaine, dibucaine, procaine and xylocaine on the rate of calcium uptake and the rate of calcium-dependent ATP splitting were examined under the conditions used for measurements of phosphoprotein formation given in Fig. 1. The rate of calcium uptake is only slightly inhibited by tetracaine and dibucaine at concentrations of 10^{-3} and $3 \cdot 10^{-4}$ M, respectively (Fig. 5). The rate of calcium-dependent ATP splitting is affected to approximately the same extent as the rate of calcium uptake at low drug concentrations, but at higher drug concentrations the inhibition of calcium uptake appears to be greater than the inhibition of the ATPase (Figs. 5 and 6). The inhibition of the rate of calcium uptake and the rate of calcium-dependent ATP splitting by sarcoplasmic reticulum vesicles is markedly enhanced at a low ionized calcium concentration (Table II), which parallels the decrease in the rate of ATP-ADP phosphate exchange and the reduction in the phosphoprotein steady-state level (Figs. 2 and 3). The inhibitory effect of procaine and xylocaine is clearly observed under these conditions. The stoichiometry between calcium uptake and ATP hydrolysis, which is about 1.6 in the controls, is not significantly altered by the drugs at the concentrations used in these experiments.

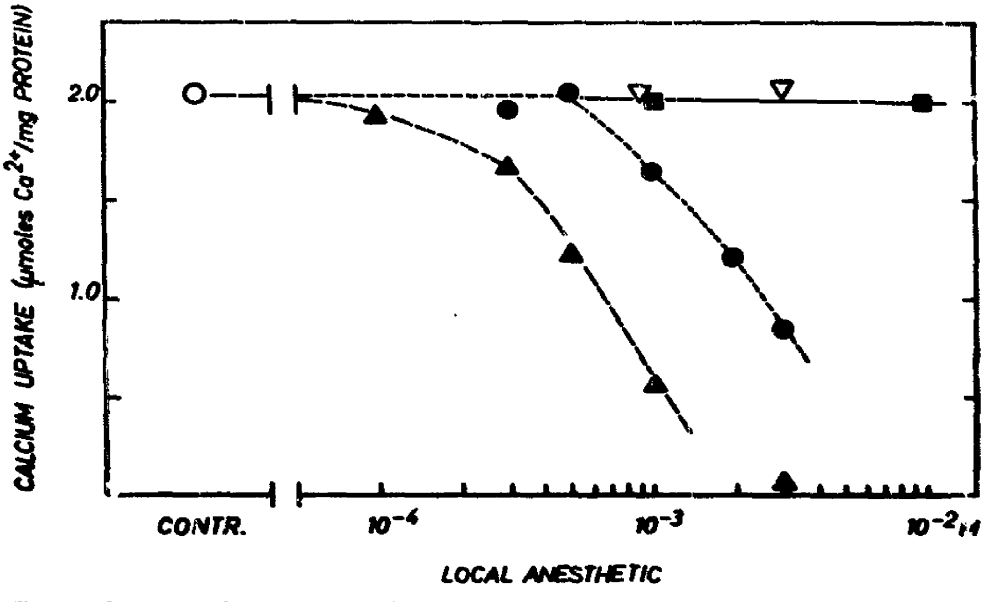


Fig. 5. Effect of various concentrations of tetracaine, dibucaine, procaine and xylocaine on calcium uptake by sarcoplasmic reticulum. Medium: 40 mM histidine buffer (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 2 mM phosphoenolpyruvate, 0.02 mg pyruvate kinase/ml, 5 mM oxalate, 0.1 mM $^{45}CaCl_2$, 0.025-0.05 mg sarcoplasmic reticulum protein/ml. $T = 25$ °C. ○—○, control; ●—●, tetracaine; ▲—▲, dibucaine; ■—■, procaine; ▽—▽, xylocaine.

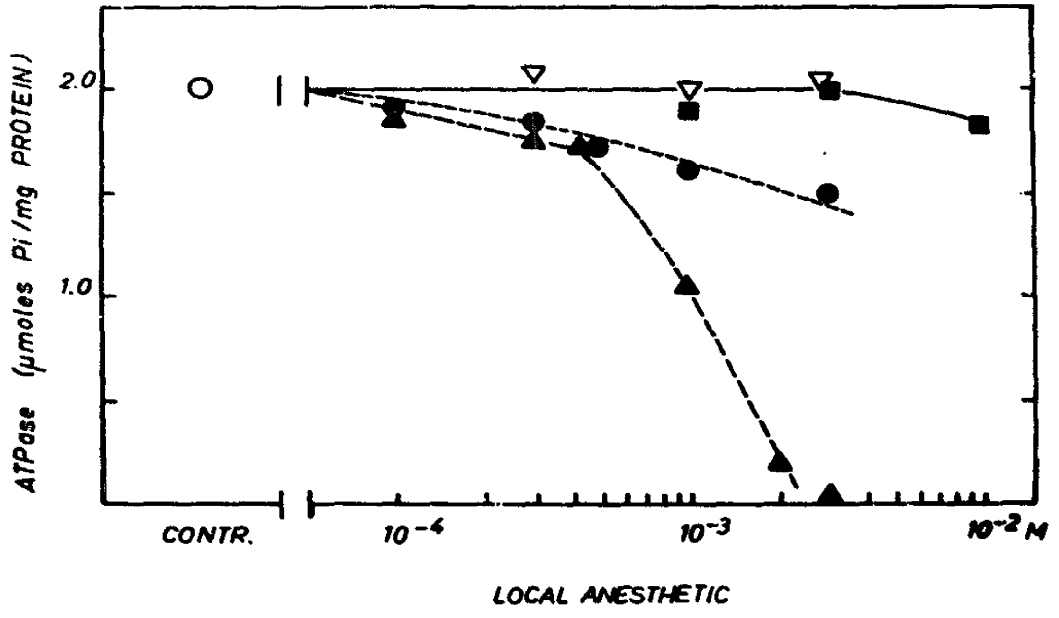


Fig. 6. Effect of tetracaine, dibucaine, procaine and xylocaine on the calcium-dependent ATP hydrolysis by the calcium ATPase of sarcoplasmic reticulum. The calcium ATPase was prepared according to MacLennan [29]. Medium: 40 mM histidine buffer (pH 7.0), 100 mM $MgCl_2$, 5 mM ATP, 2 mM phosphoenolpyruvate, 0.02 mg pyruvate kinase/ml, 0.1 mM $CaCl_2$, 0.05 mg sarcoplasmic reticulum protein/ml. $T = 25$ °C. ○—○, control; ●—●, tetracaine; ▲—▲, dibucaine; ■—■, procaine; ▽—▽, xylocaine.

TABLE II

EFFECT OF DIBUCAINE, TETRACAINE, XYLOCAINE AND PROCAINE ON THE RATE OF CALCIUM UPTAKE AND THE RATE OF CALCIUM-DEPENDENT ATP HYDROLYSIS BY SARCOPLASMIC RETICULUM

Medium: 40 mM histidine-buffer, 40 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 2 mM phosphoenolpyruvate, 0.02 mg pyruvate kinase/ml, 0.05–0.10 mg sarcoplasmic reticulum protein/ml, 0.2 mM total $^{45}CaCl_2$ and 0.4 mM EGTA; pH 7.0; $T = 25$ °C. Transport ratio, rate of calcium uptake divided by the rate of calcium-dependent ATP splitting. Values are means ±S.E. for the number of experiments given in parentheses (n).

	Drug concentration (M)	n	Ca^{2+} uptake (μmol Ca^{2+}/mg protein per min)	Ca^{2+}-ATPase (μmol P_i/mg protein per min	Transport ratio
Control	—	9	0.622±0.043	0.380±0.029	1.65±0.06
Dibucaine	$3 \cdot 10^{-4}$	3	0.310±0.041	0.201±0.030	1.56±0.06
Tetracaine	10^{-3}	4	0.274±0.015	0.167±0.009	1.63±0.06
Xylocaine	$3 \cdot 10^{-3}$	3	0.364±0.017	0.226±0.015	1.62±0.08
Procaine	10^{-2}	4	0.32 ±0.030	—	—

The passive calcium binding by sarcoplasmic reticulum assayed in the absence of ATP by equilibrium dialysis (ref. 42; see Materials and Methods) is actually reduced by dibucaine and tetracaine. Calcium binding was 16.85±1.47 and 18.74±2.03 nmol Ca^{2+}/mg sarcoplasmic reticulum protein in the presence of 10^{-3} M dibucaine, respectively, compared to 21.06±0.97 nmol/mg protein obtained in the absence of the drugs (means±S.E. of three sarcoplasmic reticulum preparations). The effects of the drugs on calcium binding are small, since the calcium concentration is rather high in these experiments, but calcium concentrations of $5 \cdot 10^{-6}$–$7 \cdot 10^{-6}$ M are present as contaminant in the medium which excludes assays of calcium binding at low calcium concentrations without EGTA or a special treatment of the solutions in order to remove the contaminating calcium.

Effect of tetracaine and dibucaine on ADP-independent calcium efflux

The rate of calcium efflux from calcium-preloaded vesicles of sarcoplasmic reticulum in the presence of EGTA, but in the absence of ADP is slightly increased by tetracaine and dibucaine at concentrations of 10^{-3} and $3 \cdot 10^{-4}$ M, respectively (Fig. 7). However, a marked elevation in the rate of calcium efflux occurs at higher drug concentrations. Similar effects are obtained in the presence of oxalate instead of orthophosphate, but the rate of calcium release is lower with calcium oxalate preloaded sarcoplasmic reticulum vesicles (Table III).

Effect of local anesthetics on ADP-dependent calcium efflux and ATP synthesis

The reversal on the calcium pump has been shown by Makinose and Hassel-

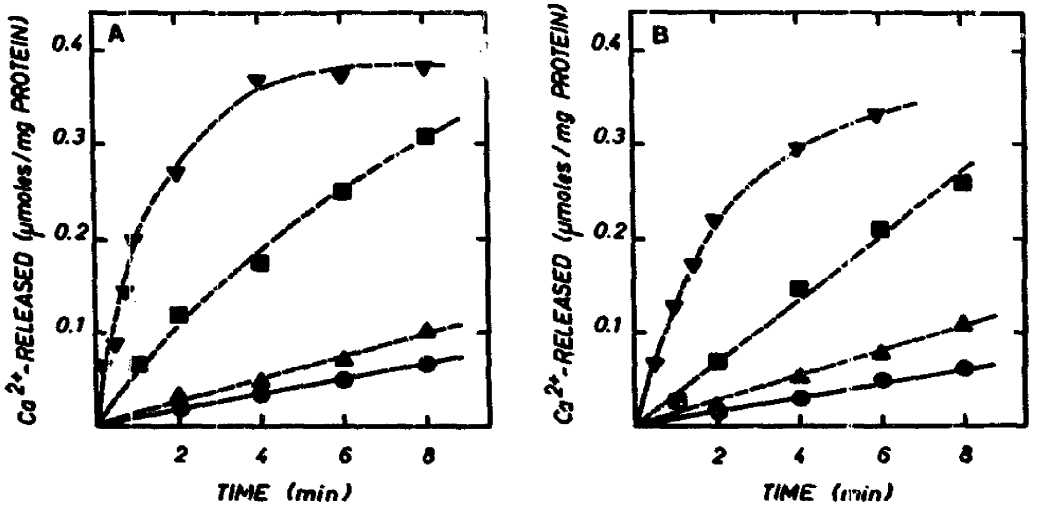


Fig. 7. The effect of tetracaine and dibucaine on ADP-independent calcium efflux from calcium preloaded sarcoplasmic reticulum vesicles in the presence of orthophosphate. Sarcoplasmic reticulum vesicles (0.5 mg protein/ml) were incubated for 25 min in a medium containing 40 mM histidine buffer (pH 7.0), 100 mM KCl, 7 mM $MgCl_2$, 2 mM acetylphosphate, 20 mM orthophosphate, 0.2 mM $^{45}CaCl_2$. T = 25 °C. At 25 min 4 mM EGTA was added. The drugs were added 15 s before EGTA. Tetracaine (A): ▲—▲, 10^{-3} M; ■—■, $2 \cdot 10^{-3}$ M; ▼—▼, $3 \cdot 10^{-3}$ M. Dibucaine (B): ▲—▲, $3 \cdot 10^{-4}$ M; ■—■, $5 \cdot 10^{-4}$ M; ▼—▼, 10^{-3} M; ●—●, control.

TABLE III

COMPARISON OF THE EFFECTS OF TETRACAINE AND DIBUCAINE ON ADP-INDEPENDENT CALCIUM EFFLUX FROM SARCOPLASMIC RETICULUM PRELOADED IN THE PRESENCE OF ORTHOPHOSPHATE OR OXALATE

Sarcoplasmic reticulum vesicles were incubated for 25 min in a medium containing 40 mM histidine buffer (pH 7.0), 100 mM KCl, 7 mM $MgCl_2$, 2 mM acetylphosphate, 0.2 mM $^{45}CaCl_2$ and 20 mM orthophosphate (A) or 5 mM oxalate (B). $T = 25$ °C. At 25 min 4 mM EGTA was added. The drugs were added 15 s before EGTA.

	Drug concentration (M)	(A) Ca^{2+} released (μmol Ca^{2+}/mg protein per min)	(B) Ca^{2+} released (μmol Ca^{2+}/mg protein per min)
Control	—	0.008	0.004
Tetracaine	$5 \cdot 10^{-3}$	0.195	0.037
Dibucaine	10^{-3}	0.125	0.030

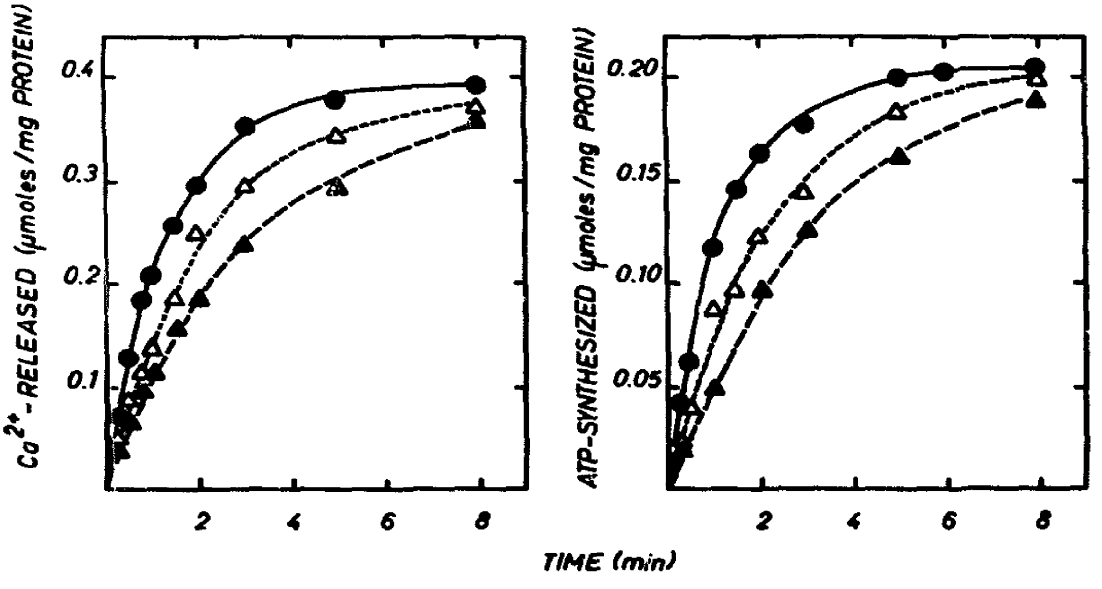


Fig. 8. Effect of tetracaine on ADP-dependent calcium efflux and ATP synthesis by sarcoplasmic reticulum. Sarcoplasmic reticulum vesicles were incubated for 25 min in a medium containing 40 mM histidine buffer (pH 7.0), 100 mM KCl, 7 mM $MgCl_2$, 2 mM acetylphosphate, 100 mM glucose, 0.5 mg sarcoplasmic reticulum protein/ml and either 20 mM orthophosphate, 0.2 mM $^{45}CaCl_2$ or 20 mM ortho[^{32}P]phosphate, 0.2 mM $CaCl_2$. $T = 25$ °C. Hexokinase (40 μg/ml) was added at 24 min. At 25 min 4 mM EGTA plus 2 mM ADP was added. Tetracaine was added 15 s before EGTA plus ADP. ● — ●, control; △ — △, $5 \cdot 10^{-4}$ M tetracaine; ▲ — ▲, 10^{-3} M tetracaine.

TABLE IV

EFFECT OF TETRACAINE AND DIBUCAINE ON ADP-INDUCED CALCIUM RELEASE AND ATP SYNTHESIS BY SARCOPLASMIC RETICULUM

Conditions as given in Fig. 8.

	Drug concentration (M)	Calcium released (μmol Ca^{2+}/mg protein per min)	ATP synthesized (μmol ATP/mg protein per min)	Ca^{2+} released / ATP synthesized
Control	–	0.251	0.118	2.12
Tetracaine	$5 \cdot 10^{-4}$	0.167	0.084	1.98
	10^{-3}	0.135	0.062	2.17
Dibucaine	10^{-4}	0.179	0.109	1.64
	$3 \cdot 10^{-4}$	0.116	0.060	1.93

bach [17], by demonstrating that the ADP-induced calcium efflux is stoichiometrically coupled with ATP synthesis. Similar data were reported by Panet et al. [20] and Yamada et al. [21, 22]. Tetracaine and dibucaine influence the ADP-induced release of calcium from calcium-preloaded vesicles in a different manner to their effect on the slow rate of calcium release obtained in the presence of EGTA, but in the absence of ADP. The rate of calcium release in the presence of 2 mM ADP and 4 mM EGTA from calcium-preloaded sarcoplasmic reticulum vesicles is reduced by about 25 and 50 % in the presence of tetracaine at concentrations of $5 \cdot 10^{-4}$ and 10^{-3} M, respectively. The rate of ATP synthesis is altered to virtually the same extent as the rate of calcium efflux, indicating that tetracaine inhibits the calcium release mediated by the ATPase protein (Fig. 8). The inhibition of the rate of calcium release and ATP

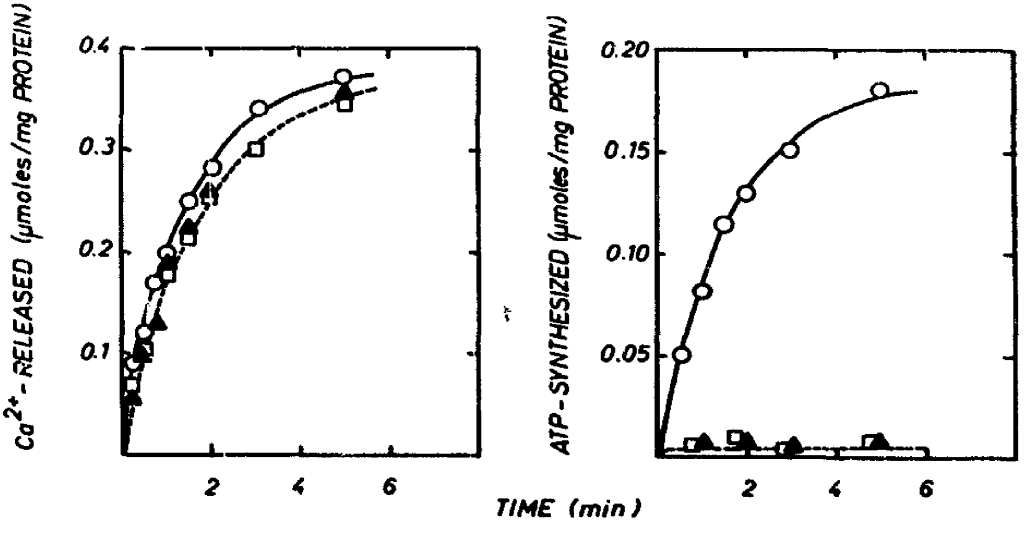


Fig. 9. Effect of "high" concentrations of tetracaine and dibucaine on calcium efflux and ATP synthesis by sarcoplasmic reticulum. Conditions as given in Fig. 8. ○—○, control; ▲—▲, $3 \cdot 10^{-3}$ M tetracaine; □—□, 10^{-3} M dibucaine.

synthesis by $3 \cdot 10^{-4}$ M dibucaine is about as great as in the presence of 10^{-3} M tetracaine. The stoichiometry between calcium release and ATP synthesis, which is about 2 [17], is unchanged by both drugs at the drug concentrations and conditions used (Table IV).

On the other hand, ADP-induced calcium release and ATP synthesis are dissociated in the presence of tetracaine and dibucaine at concentrations of $3 \cdot 10^{-3}$ and 10^{-3} M, respectively; calcium efflux appears very little affected, whilst ATP synthesis is severely depressed (Fig. 9).

Effect of local anesthetics on phosphoprotein formation by sarcoplasmic reticulum from ortho[^{32}P]phosphate

The phosphoprotein steady-state level obtained by phosphorylation of sarcoplasmic reticulum vesicles with ortho[^{32}P]phosphate is markedly reduced in the presence of increasing concentrations of tetracaine or dibucaine (Fig. 10). Little change in the phosphoprotein steady-state level is observed when the reaction is stopped either 5 s or 1 min after the addition of EGTA and/or the addition of the

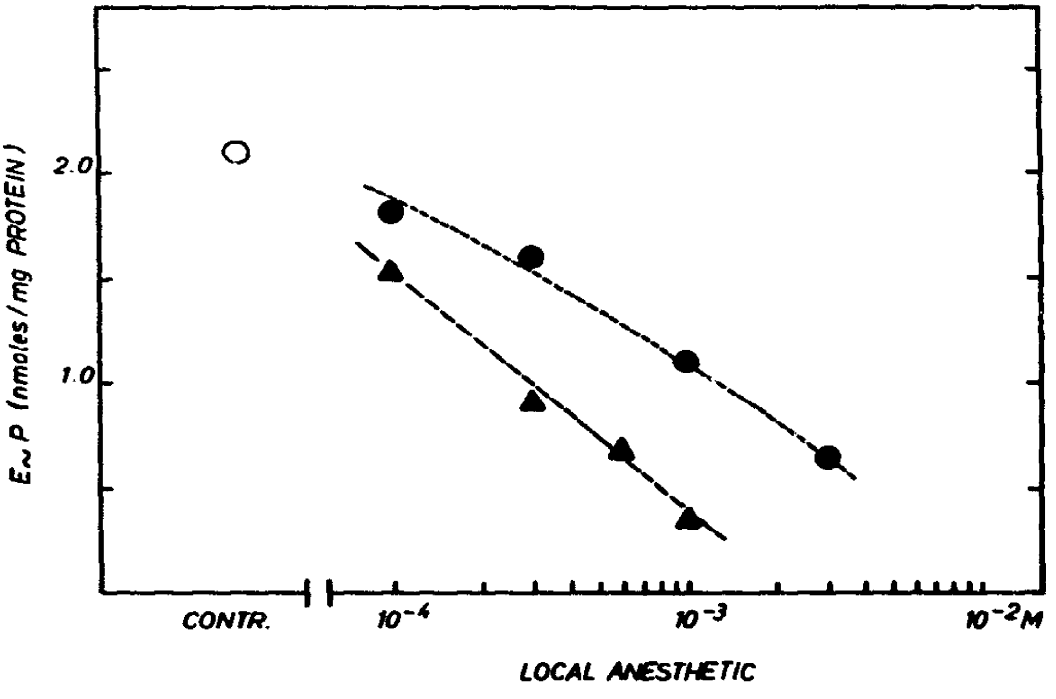


Fig. 10. Effect of tetracaine and dibucaine on phosphoprotein formation by sarcoplasmic reticulum from ortho[^{32}P]phosphate. Sarcoplasmic reticulum vesicles were incubated for 25 min in a medium containing 40 mM histidine buffer (pH 7.0), 100 mM KCl, 7 mM $MgCl_2$, 2 mM acetylphosphate, 20 mM ortho[^{32}P]phosphate, 0.2 mM $CaCl_2$, 0.5 mg sarcoplasmic reticulum protein/ml. At 25 min 2–4 mM EGTA was added. The drugs were added 5 s after EGTA and the reaction stopped 15 s later by adding a solution of 0.1 M $HClO_4$ containing 20 mM orthophosphate. The protein was recovered by centrifugation and the pellet was washed thrice with 0.1 M $HClO_4$ and 20 mM unlabelled orthophosphate and four times with a 0.1 M $HClO_4$, 200 mM NaCl solution. ○—○, control; ●—●, tetracaine; ▲—▲, dibucaine.

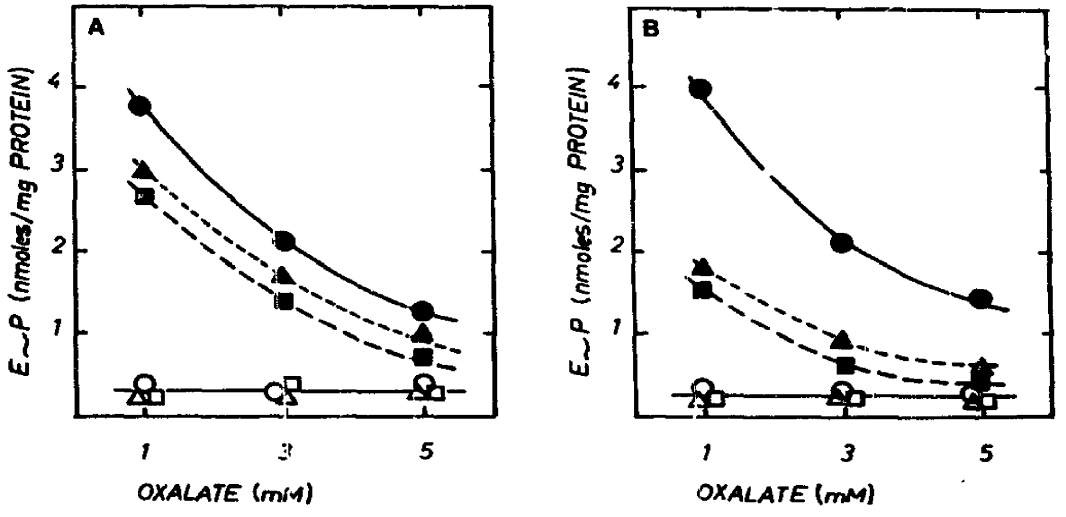


Fig. 11. Effect of tetracaine and dibucaine on phosphoprotein formation by sarcoplasmic reticulum from ortho[^{32}P]phosphate at various oxalate concentrations. Sarcoplasmic reticulum vesicles were incubated for 20 min in a medium containing 40 mM histidine buffer (pH 7.0), 100 mM KCl, 7 mM $MgCl_2$, 2 mM acetylphosphate, 0.2 mM $CaCl_2$, 0.5 mg sarcoplasmic reticulum protein/ml and 1, 3, or 5 mM oxalate. At 20 min 4 mM EGTA and 20 mM ortho[^{32}P]phosphate was added and the reaction stopped 20 s later by a solution of 0.1 M $HClO_4$ containing 20 mM unlabelled orthophosphate. The drugs were added 5 s after the addition of EGTA plus ortho[^{32}P]phosphate and the reaction was stopped as in the controls 20 s after the addition of EGTA plus ortho[^{32}P]phosphate. 2 mM ADP was added to control samples and samples with the drugs 20 s after the addition of EGTA plus ortho[^{32}P]phosphate and the reaction was stopped 5 s later. Closed symbols: without ADP; open symbols: 2 mM ADP. (A): ▲—▲, 10^{-3} M tetracaine; ■—■, $3 \cdot 10^{-4}$ M dibucaine; ●—●, control. (B): ▲—▲, $3 \cdot 10^{-3}$ M tetracaine; ■—■, 10^{-3} M dibucaine; ●—●, control.

drugs. Phosphoprotein steady-state levels are of about the same order whether the drugs are given before or after the addition of EGTA. When the intravesicular ionized calcium concentration is altered by using various concentrations of oxalate for the calcium loading of sarcoplasmic reticulum vesicles [10, 11, 28, 33, 43] a marked reduction in ortho[^{32}P]phosphate incorporation is observed in the presence of higher oxalate concentrations, i.e. at lower intravesicular free calcium concentrations (Fig. 11). In the presence of higher oxalate concentrations there is a much greater reduction in the phosphoprotein steady-state level by the drugs compared to the controls. Following the addition of ADP there is an immediate decline in phosphoprotein to a very low concentration, which is independent of the presence or the absence of the drugs. These findings seem to suggest that the dependence of phosphoprotein formation on intravesicular calcium is shifted to higher calcium concentrations in the presence of the drugs.

DISCUSSION

Local anesthetics markedly influence the forward reactions [7–15] of the calcium pump of sarcoplasmic reticulum membranes mediated by phosphoprotein

formed in the presence of calcium, magnesium and ATP in the medium, as well as the reverse reactions of the calcium pump [17–23], which depend on calcium inside the vesicles, magnesium and the products of the forward reactions, ADP and orthophosphate.

The inhibition of the rates of calcium uptake, calcium-dependent ATP splitting and ATP-ADP phosphate exchange by tetracaine and dibucaine are at least partially due to effects of both drugs on phosphoprotein formation from ATP, which seems to be reduced on account of a reduction in the apparent calcium affinity of sarcoplasmic reticulum membranes, as indicated by the alteration in calcium dependence of phosphoprotein formation and ATP-ADP phosphate exchange (Figs. 2 and 3). This interpretation is in agreement with the passive calcium binding to sarcoplasmic reticulum membranes. The calcium binding obtained in the absence of ATP is significantly reduced in the presence of dibucaine.

Martonosi et al. [4] have suggested that the inhibition of calcium uptake and calcium ATPase by tetracaine and dibucaine is due to the inhibition of dephosphorylation of the phosphorylated intermediate formed from ATP during calcium uptake. It cannot be deduced from our data whether or not the drugs diminish the dephosphorylation of the phosphoenzyme in addition to their inhibitory effects on phosphoprotein formation. Measurements of the rate of dephosphorylation in the presence or absence of the drugs by a quench-flow apparatus [23], which is not available to us at present, could answer this question.

The increase in the rate of ATP-ADP phosphate exchange observed in the presence of tetracaine at higher calcium concentrations (Figs. 3 and 4) is associated with a decrease in the ATP-splitting reaction, whilst the phosphoprotein steady state level is little affected under these conditions (Fig. 1, Table I). The decline in the rate of ATP hydrolysis is too small to account for the elevated rate of phosphate exchange. The apparent increase in the rate of ATP-ADP phosphate exchange by sarcoplasmic reticulum in the presence of tetracaine at saturating calcium concentrations as compared with the maximum rate of phosphate exchange obtained in the absence of the drug (Fig. 3) appears to be a consequence of a shift in substrate inhibition, i.e. inhibition of the phosphate exchange reaction by calcium occurs at higher ionized calcium concentration in the presence of tetracaine than in the absence of the drug.

The reduction in the rate of ADP-induced calcium release, as well as in the rate of ATP synthesis by tetracaine and nupercaine indicates that the drugs influence calcium efflux mediated by the calcium-translocating ATPase of sarcoplasmic reticulum membranes (Fig. 8 and Table IV). The inhibition of both the rate of calcium release and the rate of ATP synthesis at low drug concentrations (Fig. 8 and Table IV) and the virtually complete inhibition of ATP synthesis by high drug concentrations (Fig. 9) are associated with a reduction in the concentration of phosphoprotein formed from orthophosphate in the presence of magnesium and intravesicular calcium (Fig. 10). This reduction in the phosphoprotein steady-state level by the drugs seems to be due to inhibition of phosphoprotein formation on account of an alteration in intravesicular calcium dependence. As judged from the use of oxalate in order to vary the intravesicular free calcium concentration [28, 43], it appears that tetracaine and dibucaine reduce the apparent calcium affinity on the inside of the membrane (Fig. 11). The reduction of orthophosphate incorporation into the ATPase protein

in the presence of oxalate does not seem to be due to a competition of oxalate and phosphate as described in the case of calcium uptake [44], since the inhibition of phosphoprotein formation was practically the same when the orthophosphate-oxalate ratio was changed from 4 to 10.

It cannot be decided from the present data whether the effect of the local anesthetics on calcium translocation via the calcium transport ATPase is mediated by a direct action of the drugs on the ATPase protein or by affecting the protein lipid interaction of the sarcoplasmic reticulum membrane due to the well-known binding to the phospholipids of the membrane [45–47] or by a combination of both.

On the other hand, the drug-induced, but ADP-independent calcium efflux from calcium-preloaded sarcoplasmic reticulum (Fig. 7; Table III) is probably due to the effect of the drugs on the membrane phospholipids. It is assumed that this type of calcium efflux does not occur via the ATPase, which would explain the apparent uncoupling of calcium efflux and ATP synthesis at higher drug concentrations (Fig. 9). Otherwise one has to assume that the drugs dephosphorylate the phosphoprotein formed from orthophosphate in the presence of intravesicular calcium in order to explain the increase in the rate of calcium efflux. This latter possibility seems rather unlikely since ATP synthesis approached zero (Fig. 9), and this ought not to be the case as judged from the rates of calcium release by ADP or high drug concentrations (Figs. 7 and 8); moreover, no effect was observed of the drugs on ADP sensitivity of the phosphoprotein formed from ATP (Table I). Thus, the drug-induced release should proceed via the membrane lipids, which would be in agreement with the observations that tetracaine and dibucaine increase the calcium permeability of liposomes [4].

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