“Local anesthetics inhibit Ca-ATPase in masticatory muscles”.

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ABSTRACT

Local anesthetics have myotoxic effects and inhibit Ca-ATPase activity and Ca transport in skeletal muscles. Such effects have not been fully elucidated in masticatory muscles. We tested the hypothesis that local anesthetics increase myoplasmic calcium in masticatory muscles by inhibiting Ca-ATPase at a concentration similar to that of dental cartridges. The effect of lidocaine and bupivacaine on Ca-ATPase from rabbit masseter and medial pterygoid muscles was tested with radioisotopic and colorimetric methods. Bupivacaine had an action similar to lidocaine on Ca-ATPase activity but less effect on calcium transport. The pre-exposure of the membranes to the anesthetics enhanced the Ca-ATPase activity in the absence of calcium ionophore, supporting their permeabilizing effect. The results demonstrate that amide-type anesthetics do not inhibit calcium binding but do reduce calcium transport and enzyme phosphorylation by ATP and suggest that the myoplasmic calcium increase induced by lidocaine and bupivacaine might promote masticatory muscles contraction and eventual rigidity.

INTRODUCTION

The sarcoplasmic reticulum Ca-ATPase is a membrane-bound protein which transports calcium ions from the myoplasm to the reticulum lumen at the expense of ATP hydrolysis, leading to muscle relaxation. It has one high affinity ATP binding site (catalytic site) and two calcium binding sites (transport sites) (Clarke *et al.*, 1989).

Lacapere & Guillain (1990) proposed a summarized enzymatic cycle of the Ca-ATPase (Appendix, Fig. 1, filled lines). In the clockwise reaction, the enzyme in its E1 form binds two calcium ions on the cytoplasmic membrane side (out) with high affinity (step 1) to form the E1Ca2 species. The phosphorylation of the enzyme by ATP (step 2, yielding E1PCa2) drives the movement of calcium to the low affinity lumenal sites (in). Calcium is then released into the reticulum lumen (step 3), which favors the hydrolysis of the phosphoenzyme E2P (step 4) to E2 completing the cycle.

Few authors have studied the Ca-ATPase from masticatory muscles (Okabe *et al.,* 1985; Sánchez *et al.*, 2004). Studies on the effect of dental drugs on the Ca-ATPase from masseter and medial pterygoid muscles have not yet been published. Dental local anesthetics have been reported to inhibit the sarcoplasmic reticulum Ca-ATPase from fast muscles (Suko *et al.*, 1976; Wolosker *et al.*, 1992; Takara *et al.,* 2000 and 2005).

Microscopic studies revealed that local anesthetics can cause ultrastructural damage in muscle fibers (Benoit & Belt, 1970, Dolwick *et al.*, 1977; Yagiela *et al.*, 1981). Zink *et al.* (2003) attributed such damage to the increase in intracellular Ca^{2+}, which might derive from Ca-ATPase dysfunction. The likely link between the sarcoplasmic reticulum Ca-ATPase function and the miotoxicity of dental local anesthetics motivated this study. We tested the hypothesis that local anesthetics increase the myoplasmic calcium in masticatory muscles by inhibiting the Ca-ATPase at a concentration similar to that of dental cartridges.

MATERIALS & METHODS

Masseter and medial pterygoid muscles were sampled from adult New Zealand rabbits (6 mos old, males, 2 kg) according to Widmer *et al.* (1997). The animal use protocol was approved by the Ethics Commission, Faculty of Dentistry, University of Buenos Aires. Sarcoplasmic reticulum membranes were isolated as sealed vesicles by Champeil *et al*. (1985). The protein concentration was measured by Lowry *et al.* (1951).

For Ca-dependent ATPase activity determinations, sarcoplasmic reticulum membranes (0.1 mg protein/mL) were incubated at 37 ^{o}C for 2 min. in 50 mmol/L MOPS (3-[Nmorpholino] propanesulfonic acid)-Tris (Tris[hydroximethyl]aminomethane) buffer (pH 7.2), 3 mmol/L ATP, 100 mmol/L KCl, 3 mmol/L $MgCl_2$, 0.1 mmol/L $CaCl_2$, 0.1 mmol/L EGTA (ethyleneglycolbis (β-aminoethyl ether)-*N,N*'-tetraacetic acid), 10 μmol/L calcimycin (calcium ionophore A23187) when mentioned, and lidocaine or bupivacaine as indicated. Reactions were stopped with 5 % trichloroacetic acid (final concentration). As the accumulation of Ca inside the vesicles inhibits the Ca-ATPase activity, the Ca ionophore calcimycin was included in the media to dissipate the Ca gradient generated by the pump. The denatured membranes were precipitated by centrifugation. [P_i] was measured in the supernatants (Baginski *et al.*, 1967) and taken as an index of the ATPase activity. When indicated, prior to incubations, the membranes (0.5 mg protein/mL) were exposed to 21 mmol/L lidocaine or bupivacaine and 50 mmol/L MOPS-Tris buffer (pH 7.2). Later, the media were diluted 1:5 in solutions without lidocaine or bupivacaine, which yielded concentrations of 4.2 mmol/L. The other reactants reached final concentrations as above. Blanks without sarcoplasmic reticulum membranes were run in parallel and subtracted from the experimental values. Ca-independent ATPase activity, measured in the absence of added $CaCl_2$, was below 5% of total ATPase activity.

ATP-dependent calcium uptake was determined with a radioisotopic technique. Sarcoplasmic reticulum vesicles (0.1 mg protein/mL) were incubated at 37 ^{o}C for 30 sec. in 3 mmol/L ATP, 100 mmol/L KCl, 3 mmol/L $MgCl_2$, 0.1 mmol/L (^{45}Ca)$CaCl_2$ (450 cpm/nmol), 0.1 mmol/L EGTA and 50 mmol/L MOPS-Tris (pH 7.2). Reactions started by addition of the membranes and stopped by filtration (Millipore filters, 0.45 μm pore size, Bedford, MA). Filters were washed with 3 mmol/L $LaCl_3$. Radioactivity retained in the filters was measured in a liquid scintillation counter. Blanks without ATP were run in parallel and subtracted from the experimental values. To test the effect of lidocaine or bupivacaine, they were included in the media at different concentrations. Their effect was also determined at different free Ca and ATP concentrations. Ca uptake in the first enzyme cycle was determined by Davidson & Berman (1988): the vesicles were incubated in the above medium without ATP and with low EGTA concentration (0.02 mmol/L). Then, 25 μmol/L ATP and 0.25 mmol/L EGTA (final concentrations), and lidocaine or bupivacaine at different concentrations were added. ATP allowed the phosphorylation of the enzyme and the transport of calcium already bound to the protein, and EGTA precluded further enzyme cycles by sequestering the activating cation.

For the determinations of the passive calcium binding to the enzyme, sarcoplasmic reticulum vesicles (0.2 mg protein/mL) were incubated at room temperature for 30 sec. in 50 mmol/L MOPS-Tris (pH 7.2), 0.1 mmol/L EGTA, (^{45}Ca)$CaCl_2$ (450 cpm/nmol) at different concentrations and without or with 30 mmol/L lidocaine or bupivacaine. The media were filtered through Millipore filters and the radioactivity retained was measured in a liquid scintillation counter. Blanks without sarcoplasmic reticulum membranes were run in parallel and subtracted from the experimental values.

Ca-ATPase activity and Ca uptake were analyzed by simulation of the kinetics of the Ca-ATPase cycle (Appendix, Fig. 1) according to Hecht *et al*. (1990), using 0.1 mg protein/mL, 3 mmol/L ATP, and 0.1 mmol/L Ca. Other simulation parameters were as in Alonso & Hecht (1990), in accordance with many authors (cf De Meis, 1981). High and low spontaneous Ca diffusion across the membrane were assumed (Appendix, Fig. 1, dotted line), simulating the presence or absence of calcimycin, and Ca-ATPase activity and Ca uptake were calculated from the respective results. The rate constants of the partial steps were adjusted to obtain Ca-ATPase activity and Ca uptake comparable to those experimentally obtained in the absence of inhibitors. Data from 10-seconds simulated reactions including various concentrations of a local anesthetic interacting with an intermediate of the cycle (Appendix, Fig. 1, dashed line), were used to calculate the inhibition constants K_i (activity) and K_i (uptake).

Disodium ATP, calcimycin, lidocaine hydrochloride, bupivacaine hydrochloride, MOPS and Tris were purchased from Sigma Chemical Co (St. Louis, MO, USA). All other reagents were analytical grade. (^{45}Ca)$CaCl_2$ was from New England Nuclear (Boston, MA).

Mean values of the results are given with the SD. Lidocaine or bupivacaine half-maximal inhibitory concentrations for Ca-ATPase activity or calcium uptake (K_i) are reported with the SEM and were tested for its significance by Student´s t test ($p < 0.05$). Equations were fitted to experimental data as reported by Fraser & Suzuki (1973). Free calcium concentrations were calculated by Fabiato & Fabiato (1979).

RESULTS

Lidocaine (Appendix, Fig. 2) inhibited the Ca-ATPase activity (Fig. 1A) and bupivacaine (Appendix, Fig. 2) had similar effect (Appendix, Fig. 4A).

In enzymatic activity determinations, K_i values for lidocaine and bupivacaine were not significantly different (Table).

The effect of lidocaine or bupivacaine on Ca-ATPase activity did not depend on the membrane protein concentration in the incubation medium (data not shown).

The Ca-ATPase activity depended on the pre-exposure time of the sarcoplasmic reticulum membranes in lidocaine (Fig. 1B) or bupivacaine (Appendix, Fig. 4B). The enzymatic activity appeared inhibited upon increasing the preincubation time when measured in the presence of calcimycin (optimal conditions), whereas appeared enhanced when measured in the absence of ionophore, a fact attributed to the increased membrane permeability. These opposing effects tended to equalize ATPase activities measured under both conditions as the incubation time approached 40 minutes. Note that final lidocaine concentration in Fig. 1B was 4.2 mmol/L, a concentration without appreciable effect in the absence of pre-exposure (Fig. 1A).

Lidocaine and bupivacaine inhibited Ca uptake in a concentration-dependent manner (Fig. 2A and Appendix, Fig. 5A). Bupivacaine K_i was significantly higher than for lidocaine (Table). K_i values for Ca uptake were higher than for Ca-ATPase activity (Table).

Calcium uptake as a function of free Ca concentration uncovered a sigmoidal profile corresponding to the calcium activation phenomenon (Fig 2B). Lidocaine decreased maximal calcium accumulation but did not affect calcium affinity. Calcium uptake as a function of ATP concentration showed a progressive and saturating increase of calcium accumulation (Fig 2C). With 30 mmol/L lidocaine the curve showed a similar shape with a considerable decrease in the absolute values. Increasing ATP could not relieve the inhibition by lidocaine or bupicavaine. The effect of the anesthetics on the phosphorylation of the enzyme by ATP was studied analyzing calcium transport during

the first enzyme cycle. The results (Fig. 2D) confirmed that lidocaine interferes with the phosphorylation reaction. Calcium binding to the enzyme (Appendix, Fig. 1, step 1) was not modified by lidocaine (Appendix, Fig. 3) or by bupivacaine (Appendix, Fig. 6). The inhibition by lidocaine and bupivacaine was not competitive with respect to the specific transport and catalytic sites of the enzyme as deduced from the Ca or ATP concentration dependence of Ca transport.

The simulations of the transport reaction yielded the evolution of the concentrations of all the species of the model. Ca-ATPase activity calculated from the rate of P_i production was 235 μmol P_i . mg protein^{-1} . hr^{-1} in the model with high Ca diffusion across the membrane. Ca uptake was 25 nmol . mg protein^{-1} in the model with low Ca diffusion, under steady state conditions. Both results were in agreement with those experimentally obtained in the absence of added local anesthetics (Figs 1A and 2A). The transient evolution of E1PCa2 and E2P species with time depended on the simulated presence or absence of calcimycin (Fig 3, A and B).

For an inhibitor interacting with E2P species (Appendix, Fig. 1), K_i (app) for Ca-ATPase activity, measured in the presence of calcimycin, was lower than K_i (app) for Ca uptake, measured in the absence of calcimycin (Fig 3C). When the inhibitor interacted with E1PCa2 species, the opposite result was obtained (data not shown).

DISCUSSION

Hydrophobic drugs inhibit Ca-ATPase (Sokolove *et al.*, 1986; Michelangeli *et al.*, 1990; Martinez-Azorin *et al.*, 1992; Caravaca *et al.*, 1995; Takara *et al.*, 2000 and 2005). Here we report that lidocaine and bupivacaine inhibit the Ca-ATPase activity in sarcoplasmic reticulum membranes from masseter and medial pterygoid muscles. The Ca-ATPase activity is affected by the previous exposure of the sarcoplasmic reticulum membranes

to lidocaine or bupivacaine. This demonstrates the dual effect of the local anesthetic. On one hand, lidocaine and bupivacaine inhibit the optimal Ca-ATPase activity measured in the presence of calcimycin. On the other hand, in the absence of ionophore, lidocaine and bupivacaine increase the membrane permeability thus enhancing the ATPase activity by precluding the inhibitory effect of intravesicular Ca accumulation.

K_i values for Ca uptake were higher than for enzymatic activity. Here we must consider that Ca-ATPase activity is measured in the presence of calcimycin and calcium uptake in its absence, and that the relative distribution of the intermediate species of the cycle during the steady state of the reactions is different under both conditions (Takara *et al*., 2005). Since the enzyme undergoes conformational changes along the cycle one could expect that any inhibitor displays different affinities for the different enzymatic conformations, and therefore, any change in the relative distribution of the conformers must affect K_i(app) of the inhibitor. The simulations of the behavior of the model under conditions reproducing the experimental results support the proposal of the interaction of the anesthetics with the E2 species.

The bupivacaine K_i for calcium uptake was significantly higher than for lidocaine. We attribute it to their different chemical structures: the bupivacaine molecule has an N-butyl piperidine group, which gives the molecule higher molar relative mass, size and steric impediment. Ropivacaine, has an N-propyl piperidine group and this difference with bupivacaine was associated with different myotoxicities (Zink *et al.*, 2003).

For diethylstilbestrol and ritodrine, the inhibition of the enzyme decreases upon increasing the protein concentration in the reaction medium. This is attributed to drug partitioning into the lipid bilayer. The inhibition of the Ca-ATPase by lidocaine and bupivacaine does not depend on the protein concentration. This is consistent with a moderate octanol/water partition coefficient (Leo *et al.*, 1971; Courtney, 1980).

Lidocaine and bupivacaine K_i in masticatory muscles are lower than those in white fast muscles (Suko *et al*., 1976, Wolosker *et al.*, 1992, Takara *et al.*, 2000 and 2005), indicating a higher affinity of the anesthetics for the Ca-ATPase from masticatory muscles. Previous assumptions on the presence of a different type of isoform in masticatory muscles (Sánchez *et al.*, 2004) could explain this fact.

The study of partial reactions of the Ca-ATPase cycle permits to elucidate the action of different compounds on this enzyme. The experiments where the enzyme was preincubated in ^{45}Ca and later ATP, EGTA and lidocaine or bupivacaine were added reflect only the transport of Ca already bound to the enzyme through steps 1, 2 and 3 of the cycle. On one hand, our results show that Ca binding to the transport sites (step 1) is not affected by lidocaine, and this result might also indicate that lidocaine does not affect step 3, and since Ca transport depends on ATP concentration but ATP does not relieve the inhibitory effect of the anesthetic, the results point to indicate that the partial reaction affected is the transference of phosphate from ATP to the enzyme. On the other hand, several authors reported a higher affinity of local anesthetics for the E2 conformers and a marked inhibition of phosphorylation through the reversal of step 4 (Suko et al, 1976; Wolosker *et al*, 1992; Takara *et al*, 2000 and 2005), and the simulations results also support a larger interaction of these drugs with the E2 conformers. Taking together both groups of results we conclude that lidocaine and bupivacaine affect both phosphorylation / dephosphorylation -steps (2 and 4) of the cycle**.**

Our results show that lidocaine and bupivacaine at concentrations present in dental cartridges inhibit the sarcoplasmic reticulum Ca-ATPase in masseter and medial pterygoid muscles. Although the inhibition of the Ca-ATPase could not fully explain

their myotoxic effects, the interaction between the anesthetics and the protein might induce masticatory muscle contraction and eventual rigidity.

ACKNOWLEDGEMENT

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LEGENDS FOR THE FIGURES

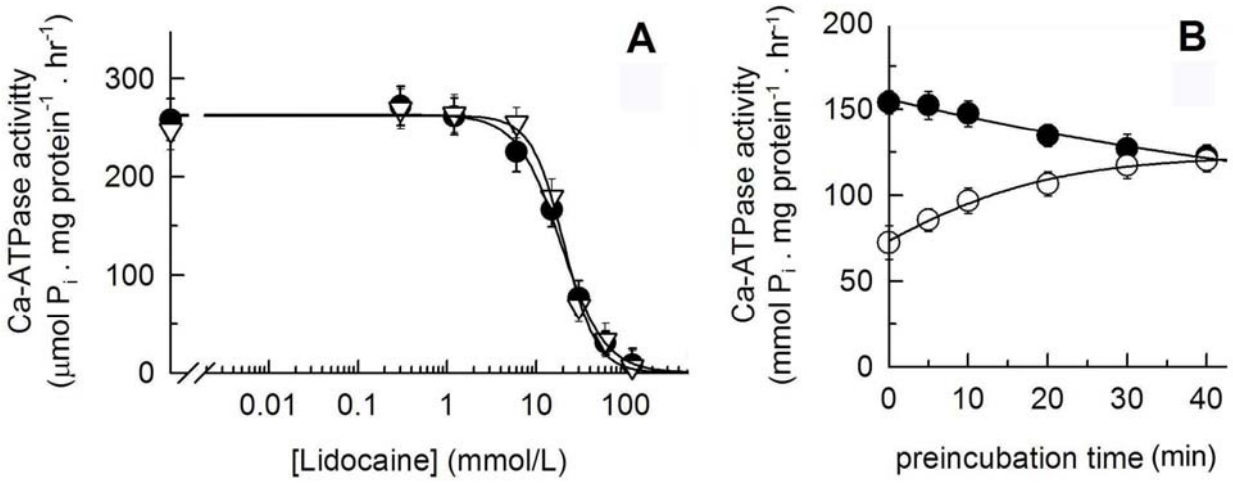
Figure 1. Ca-ATPase activity. Bars indicate the SD; n = 4. (A) Influence of lidocaine concentration on sarcoplasmic reticulum membranes derived from masseter (•) and medial pterygoid (∇) muscles. (B) Effect of lidocaine (21 mmol/L) pre-exposure time on Ca-ATPase activity (masseter sarcoplasmic reticulum in the presence (•) or absence (o) of calcimycin).

Figure 2. Calcium uptake. Bars indicate the SD; n = 4. (A) Influence of lidocaine concentration on sarcoplasmic reticulum vesicles derived from masseter (•) and medial pterygoid (∇) muscles. (B) Effect of free Ca concentration (expressed as the pCa) on Ca uptake (masseter sarcoplasmic reticulum) in the presence of 0 (•), 21 (Δ), or 30 (o) mmol/L lidocaine. (C) Influence of ATP concentration on Ca uptake (masseter sarcoplasmic reticulum) in the presence (o) or absence (•) of 30 mmol/L lidocaine. (D) Effect of lidocaine concentration on Ca transport during the first enzymatic cycle in sarcoplasmic reticulum vesicles derived from masseter (•) and medial pterygoid (∇) muscles.

Figure 3. Numerical simulations in conditions of high (•) and low (o) Ca^{2+} diffusion (K_D (diffusion constant) = 1000 s^{-1} and 3 s^{-1}, respectively). (A) Transient evolution of the E1PCa2 species of Ca-ATPase. (B) Transient evolution of the E2P species. (C) Calculated Ca-ATPase and Ca uptake activities after simulated 10-second reactions as a function of lidocaine concentration in which the lidocaine inhibitor is assumed to bind to the E2P species with a dissociation constant (K_d) of 4.5 mmol/L. Results are expressed as percentages of values obtained in the absence of lidocaine.

Table. Anesthetics concentrations for half maximal inhibition (K_i) of Ca-ATPase activity and Ca uptake in masticatory muscles. K_i values are reported as mean ± SEM. Comparisons between data sharing the same superscript ($^{a,\ b,\ c,\ d,\ e}$) are significantly different ($p < 0.05$).

Figure 1


A
Ca-ATPase activitty
(μmol P_i . mg protein^{-1} . hr^{-1})
300
200
100
0
0.01
0.1
1
10
100
[Lidocaine] (mmol/L)
B
Ca-ATPase activity
(mmol P_i . mg protein^{-1} . hr^{-1})
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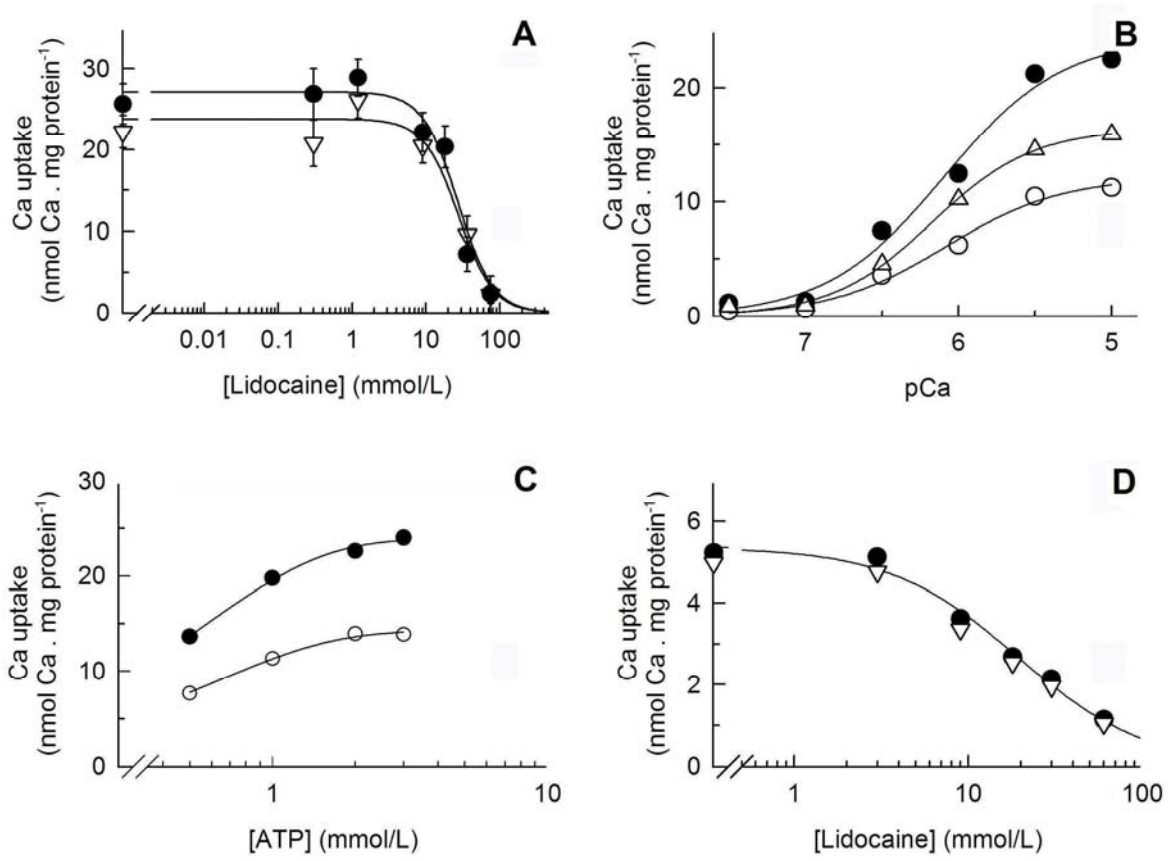
150
100
50
0
0
10
20
30
40
preincubation time (min)

Figure 2


A
Ca uptake
(nmol Ca . mg protein-1)
30
20
10
0
0.01
0.1
1
10
100
[Lidocaine] (mmol/L)
B
Ca uptake
(nmol Ca . mg protein-1)
20
10
0
7
6
5
pCa
C
Ca uptake
(nmol Ca . mg protein-1)
30
20
10
0
1
10
[ATP] (mmol/L)
D
Ca uptake
(nmol Ca . mg protein-1)
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4
2
0
1
10
100
[Lidocaine] (mmol/L)

Figure 3

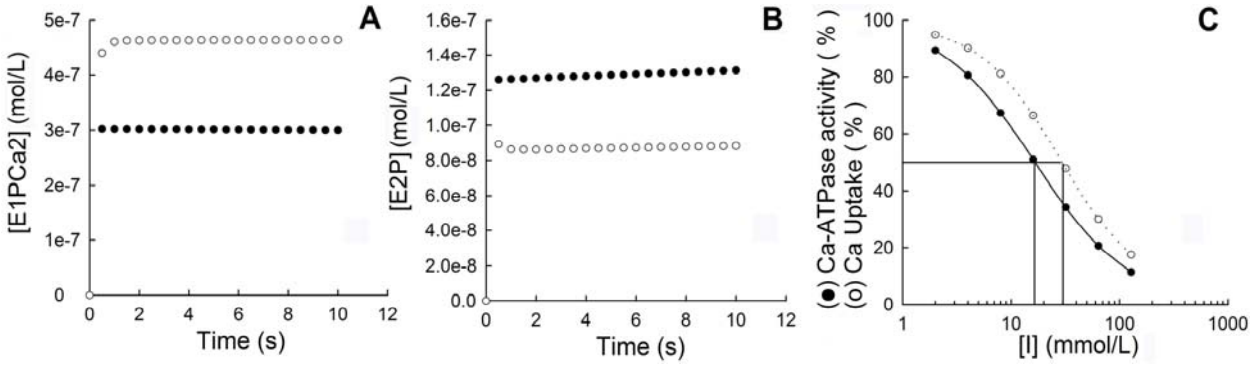

A
[E1PCa2] (mol/L)
5e-7
4e-7
3e-7
2e-7
1e-7
0
0 2 4 6 8 10 12
Time (s)
B
[E2P] (mol/L)
1.6e-7
1.4e-7
1.2e-7
1.0e-7
8.0e-8
6.0e-8
4.0e-8
2.0e-8
0.0
0 2 4 6 8 10 12
Time (s)
C
(●) Ca-ATPase activity (%)
(o) Ca Uptake (%)
100
80
60
40
20
0
1 10 100 1000
[I] (mmol/L)

Table 1

	Lidocaine	Bupivacaine	Muscle
Ca-ATPase activity	19.31 ± 1.87 (n = 6)	19.30 ± 1.12[c] (n = 5)	Masseter
ATP-dependent calcium uptake	25.10 ± 2.95[a] (n = 5)	38.12 ± 2.81[a,c] (n = 4)	
Ca-ATPase activity	20.03 ± 1.36[d] (n = 6)	22.60 ± 2.18[e] (n = 5)	Medial pterygoid
ATP-dependent calcium uptake	27.07 ± 1.24[b,d] (n = 5)	38.86 ± 2.72[b,e] (n = 4)	

Supplementary appendix
"Local anesthetics inhibit the Ca-ATPase in masticatory muscles".

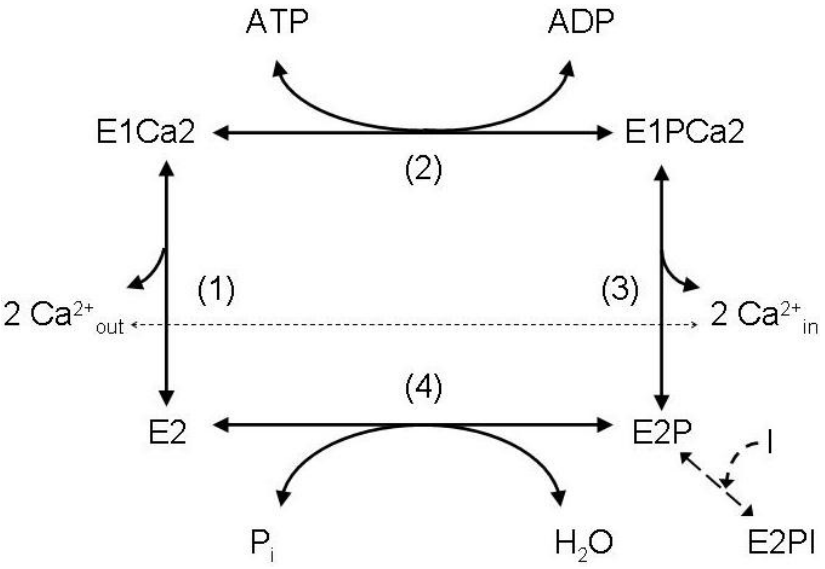
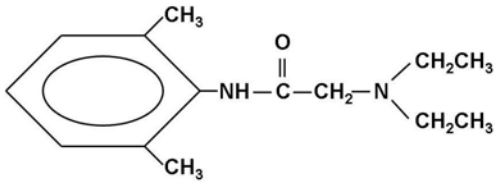
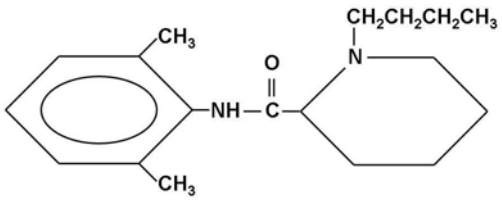


Figure 1. Minimal sequence of reactions of the catalytic cycle of the Ca-ATPase.



LIDOCAINE



BUPIVACAINE

Figure 2. Chemical structure of lidocaine and bupivacaine.

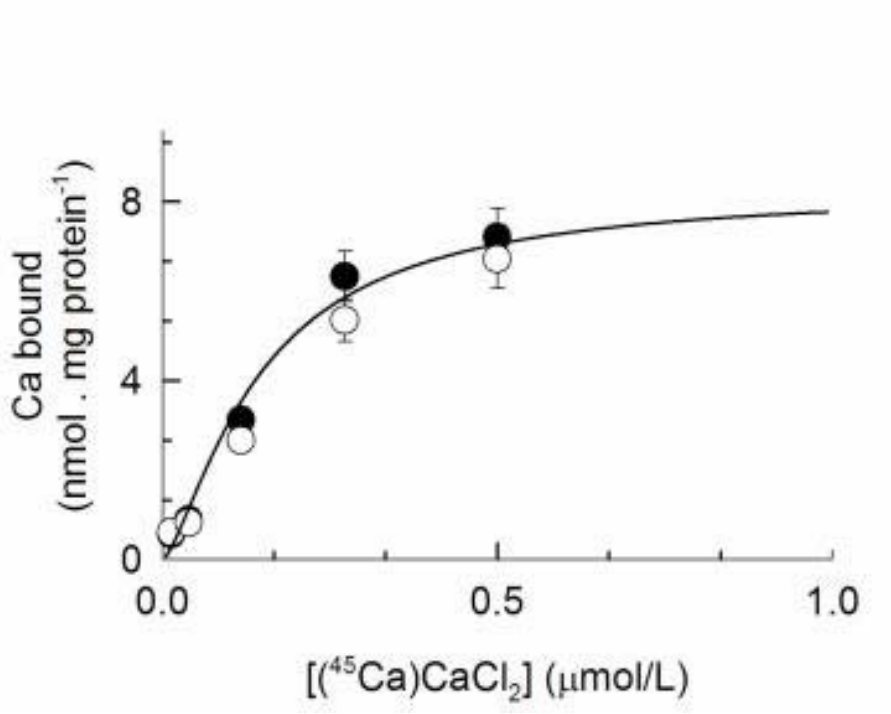


Figure 3. Calcium binding. Effect of 30 mmol/L lidocaine (o) on calcium binding in sarcoplasmic reticulum membranes from masseter muscle. (•): untreated controls**.** Bars indicate the SD, n = 4.

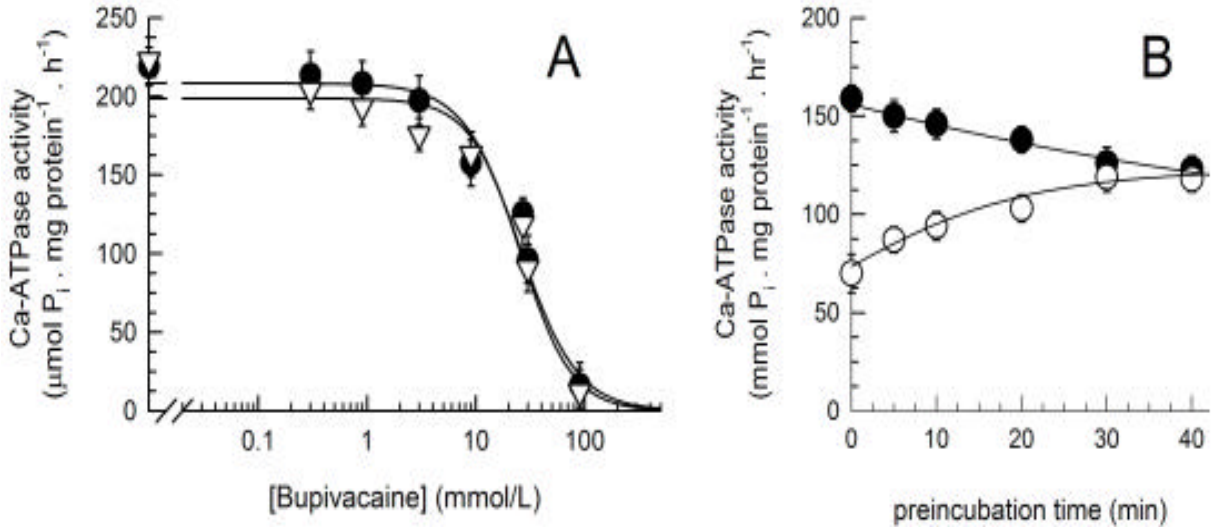


Figure 4. Ca-ATPase activity. Bars indicate the SD; n = 4. (A) Influence of bupivacaine concentration on sarcoplasmic reticulum membranes derived from the masseter (•) and medial pterygoid (∇) muscles. (B) Effect of bupivacaine (21 mmol/L) pre-exposure time on Ca-ATPase activity (masseter sarcoplasmic reticulum in the presence (•) or absence (o) of calcimycin).

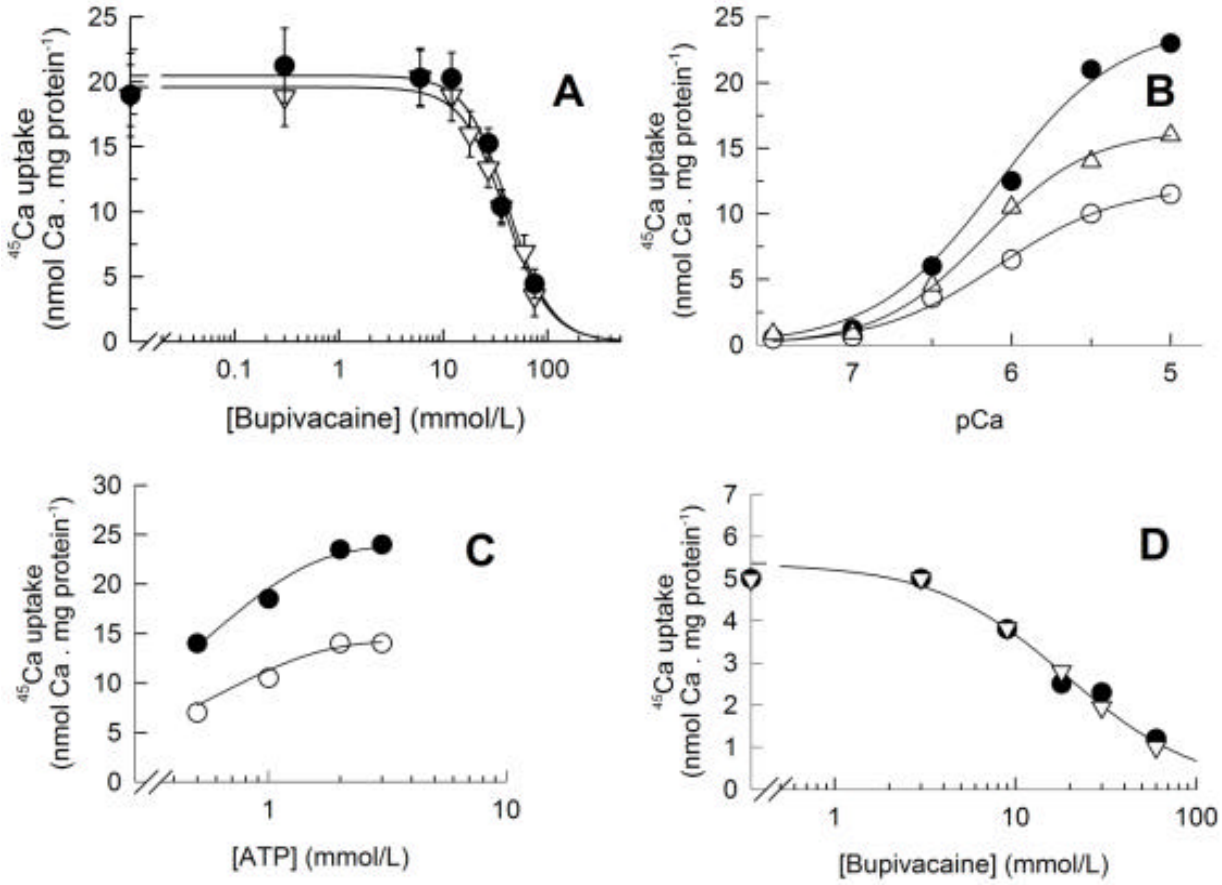


Figure 5. Calcium uptake. (A) Influence of bupivacaine concentration on sarcoplasmic reticulum vesicles derived from the masseter (•) and medial pterygoid (∇) muscles. Bars indicate the SD; n = 4. (B) Effect of free Ca concentration (expressed as the pCa) on Ca uptake (masseter sarcoplasmic reticulum) in the presence of 0 (•), 21 (∆), or 30 (o) mmol/L bupivacaine. (C) Influence of ATP concentration on Ca uptake (masseter sarcoplasmic reticulum) in the presence (o) or absence (•) of 30 mmol/L bupivacaine. (D) Effect of bupivacaine concentration on Ca transport during the first enzymatic cycle in sarcoplasmic reticulum vesicles derived from the masseter (•) and medial pterygoid (∇) muscles.

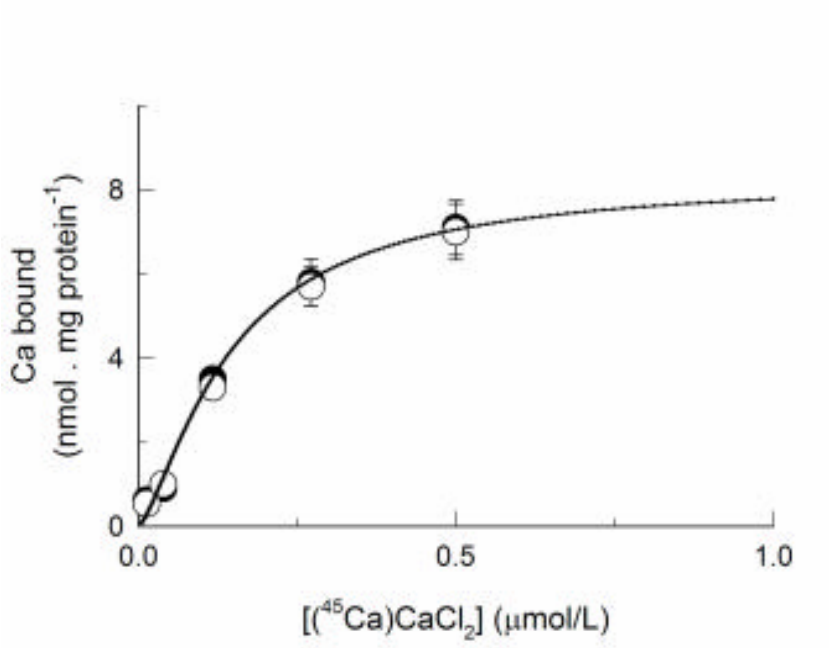


Figure 6. Calcium binding. Effect of 30 mmol/L bupivacaine (o) on calcium binding in sarcoplasmic reticulum membranes from masseter muscle. (•): untreated controls**.** Bars indicate the SD, n = 4.