INHIBITORY EFFECT OF LIDOCAINE ON THE SARCOPLASMIC RETICULUM Ca^{2+}- DEPENDENT ATPASE FROM *TEMPORALIS* MUSCLE

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ABSTRACT

Myotoxic effects of local anesthetics on skeletal muscle fibers involve the inhibition of sarcoplasmic reticulum Ca^{2+}-dependent ATPase activity and Ca^{2+} transport. Lidocaine is a local anesthetic frequently used to relieve the symptoms of trigeminal neuralgia. The aim of this work was to test the inhibitory and/or stimulatory effect of lidocaine on sarcoplasmic reticulum Ca^{2+}-dependent ATPase isolated from rabbit temporalis muscle. Ca^{2+}-dependent ATPase activity was determined by a colorimetric method. Calcium-binding to the Ca^{2+}-dependent ATPase, Ca^{2+} transport, and phosphorylation of the enzyme by ATP were determined with radioisotopic techniques. Lidocaine inhibited the Ca^{2+}-dependent ATPase activity in a concentration-dependent manner. The preincubation of the sarcoplasmic reticulum membranes with lidocaine enhanced the Ca^{2+}-dependent ATPase activity in the absence of calcium ionophore. Lidocaine also inhibited both Ca^{2+} uptake and enzyme phosphorylation by ATP but had no effect on Ca^{2+}-binding to the enzyme. We conclude that the effect of lidocaine on the sarcoplasmic reticulum Ca^{2+}-dependent ATPase from temporalis muscle is due to the drug's direct interaction with the enzyme and the increased permeability of the sarcoplasmic reticulum membrane to Ca.

Key words: sarcoplasmic reticulum; Ca^{2+}-dependent ATPase; temporal muscle; local anesthetics; lidocaine; calcium transport.

EFECTO INHIBITORIO DE LA LIDOCAÍNA SOBRE LA CALCIO ATPASA DEL RETÍCULO SARCOPLÁSMICO DEL MÚSCULO TEMPORAL

RESUMEN

La toxicidad de los anestésicos locales sobre las fibras musculares esqueléticas involucra a la inhibición de la actividad de la calcio ATPasa del retículo sarcoplásmico y a la inhibición del transporte del calcio. Tales efectos inhibitorios no han sido aún descriptos en el músculo temporal. La lidocaína es un anestésico local habitualmente usado para aliviar los síntomas de la neuralgia del trigémino por medio de la anestesia infiltrativa de la región temporal. El objetivo del trabajo fue demostrar el efecto inhibitorio y/o activador de la lidocaína sobre la calcio ATPasa del retículo sarcoplásmico del músculo temporal del conejo. La actividad de la calcio ATPasa se determinó empleando un método colorimétrico. La unión del calcio a la enzima, el transporte del calcio y la fosforilación de la ATPasa por ATP se determinaron mediante el empleo de técnicas radioisotópicas. La lidocaína inhibió a la actividad de la calcio ATPasa. El efecto inhibitorio incrementó en función de la concentración del anestésico. La preincubación de las membranas del retículo sarcoplásmico en lidocaína incrementó la actividad de la calcio ATPasa en ausencia de un ionóforo de calcio. Tal resultado avala el efecto permeabilizante del anestésico local sobre las membranas del retículo sarcoplásmico del músculo temporal. La lidocaína inhibió la captación del calcio y la fosforilación de la calcio ATPasa por ATP, pero no evidenció efecto sobre la unión del calcio a la enzima. Concluimos que el efecto de la lidocaína sobre la calcio ATPasa del retículo sarcoplásmico del músculo temporal se debe a la acción directa de la droga sobre la enzima y al incremento inducido de la permeabilidad de la membrana del retículo sarcoplásmico al Ca.

Palabras clave: retículo sarcoplásmico, Ca^{2+}-ATPasa, músculo temporal, anestésico local, lidocaína, transporte de calcio.

INTRODUCTION

The sarcoplasmic reticulum (SR) Ca^{2+}-dependent ATPase is a membrane-bound protein responsible for active Ca^{2+} accumulation during muscle relaxation. The essential role of this enzyme in skeletal muscles is to keep myoplasmic Ca^{2+} concentration low[1,2]. The ATPase has one high affinity ATP binding site (catalytic site) and two high affinity Ca^{2+} binding sites (transport sites)[3,4]. Lacapere and Guillain[5] proposed an enzymatic cycle of the Ca^{2+}-dependent ATPase in which the two main enzymatic conformations of the cycle are known as E_1 and E_2. In the forward direction of the reaction cycle, E_1 binds two Ca^{2+} (Step 1) and it is subsequently phosphorylated by ATP (Step 2). Thus, it drives the movement of Ca^{2+} from the myoplasm to the SR lumen, allowing the translocation of the cation across the SR membrane (Step 3). In the backward direction, the ATPase (E_2) is phosphorylated by inorganic phosphate (P_i) (Step 4) and the energy derived from the calcium gradient is used by the enzyme to synthesize ATP from ADP and P_i[6].

Ca^{2+}-dependent ATPase activity and Ca^{2+} transport has been previously reported in some masticatory muscles[7-9], but not in *temporalis*, a main jaw-closing muscle involved in a variety of oral functions. Lidocaine is an amide-type local anesthetic frequently used to relieve the acute symptoms of trigeminal neuralgia through infiltrative anesthesia of the temporal region[10]. Moreover, some local anesthetics decrease Ca^{2+} uptake[11-14] and increase Ca^{2+} efflux[14-17] through Ca^{2+}-dependent ATPase in fast skeletal muscles, but studies on the Ca^{2+}-dependent ATPase activity measured in the presence of Ca^{2+} ionophore are lacking[14]. The diffusion of local anesthetics into muscle fibers might trigger undesired effects such as the inhibition of the Ca^{2+} pump and the consequent increase in myoplasmic Ca^{2+} concentration. Since the sarcoplasmic reticulum Ca^{2+}-dependent ATPase is one of the myoplasm Ca^{2+}-removing systems involved in muscle relaxation, the alteration of the enzyme function by local anesthetics might be responsible for the side effect of these drugs[18,19]. Some pathological conditions, such as the sustained muscle contraction could be associated with the action of local anesthetics.

The aim of this work was to determine the inhibitory and/ or stimulatory effect of lidocaine on the sarcoplasmic reticulum Ca^{2+}-dependent ATPase from *temporalis* muscle. We tested the hypothesis that lidocaine inhibits or stimulates some steps of the sarcoplasmic reticulum Ca^{2+}-dependent enzymatic cycle.

MATERIALS AND METHODS

Membrane Preparation. *Temporalis* muscles were sampled from adult New Zealand rabbits (6 months old, males, 2 kg) for the isolation of SR membranes as sealed vesicles by centrifugation[20]. The protein concentration was measured by Lowry et al.[21]. The National Institute of Health guidelines for the care and use of laboratory animals were observed. The animal use protocol was reviewed and approved by the Ethics Commission, School of Dentistry, University of Buenos Aires.

Ca^{2+}-dependent ATPase Activity. SR membranes (0.1 mg/ml) were incubated at 37°C for 2 min in 50 mM MOPS-Tris buffer (pH 7.2), 10 µM calcimycin (calcium ionophore A23187), 3 mM ATP, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM EGTA and lidocaine at various concentrations. Since Ca^{2+} accumulation inside the vesicles inhibits Ca^{2+}-dependent ATPase activity, calcimycin was added to dissipate the Ca^{2+} gradient generated by the ATPase. Reactions were stopped with 5% trichloroacetic acid (final concentration). The denatured protein was precipitated by centrifugation and inorganic phosphate was measured in the supernatants[22] and taken as an index of the ATPase activity. When indicated, prior to incubations, the membranes (0.5 mg protein/ml) were exposed to 50 mM MOPS-Tris buffer (pH 7.2) and 21 mM lidocaine (concentration for half-maximal inhibition (K_i) of Ca^{2+}-dependent ATPase activity). Later, the media were diluted 1:5 in solutions without lidocaine. The other reagents reached final concentrations as above. Blanks without SR membranes were run in parallel and subtracted from the experimental values.

ATP-dependent Calcium Uptake. SR membranes (0.1 mg/ml) were incubated at 37°C for 30 sec in 3 mM ATP, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM (^{45}Ca)$CaCl_2$ (450 cpm/nmol), 0.1 mM EGTA, 50 mM MOPS-Tris buffer (pH 7.2) and lidocaine at different concentrations. Reactions were stopped by filtration (Millipore filters, 0.45 µm pore size, Bedford, MA, USA). Filters were immediately washed with cold 3 mM $LaCl_3$. The 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. The effect of lidocaine on ATP-dependent Ca^{2+} uptake was also determined at different free Ca^{2+} and ATP concentrations. Lidocaine concentrations for half-maximal inhibition (K_i) of Ca^{2+}-dependent ATPase activity (21 mM) and Ca^{2+} uptake ($\cong$ 30 mM) were used. Free Ca^{2+} concentrations were calculated by Fabiato & Fabiato[23]. The Ca^{2+} transport in a single enzyme turnover at different lidocaine concentrations was measured as described by Davidson & Berman[24].

Passive Ca^{2+}-binding to the Enzyme. SR vesicles (0.2 mg/ml) were incubated at room temperature for 30 sec in 50 mM MOPS-Tris buffer (pH 7.2), 0.1 mM EGTA, (^{45}Ca)$CaCl_2$ (450 cpm/nmol) at different concentrations and without or with 30 mM lidocaine. The media were filtered through Millipore filters and the radioactivity retained was measured in a liquid scintillation counter. Blanks without SR membranes were run in parallel and subtracted from the experimental values.

Chemicals and Radioisotopes. Disodium ATP (adenosine triphosphate), calcimycin, bovine seroalbumin, lidocaine, MOPS (3-[n-morpholino]propanesulfonic acid) and Tris (Tris[hydroximethyl]aminomethane) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade. (^{45}Ca)$CaCl_2$ was from New England Nuclear (E.I. Dupont de Nemours, Boston, MA, USA). The radioisotope use protocol was reviewed by the National Commission of Atomic Energy, Argentina.

Data Presentation and Statistical Analysis. Mean values of the results are given with the SD. The half-maximal concentrations of lidocaine that inhibit the Ca^{2+}-dependent ATPase activity or calcium uptake (K_i) are reported with the SEM. The difference in K_i values was tested for its significance by Student's *t* test. The level of significance was $p<0.05$.

RESULTS

Lidocaine inhibited the Ca^{2+}-dependent ATPase activity in a concentration-dependent manner (Fig. 1A, Table 1). Some additional information was obtained by repeating the experiments in the presence of different membrane protein concentrations. The inhibitory effect of lidocaine did not depend on this parameter (data not shown).

The Ca^{2+}-dependent ATPase activity varied with the pre-incubation time of the sarcoplasmic reticulum membranes from *temporalis* muscle with 21 mM lidocaine (Fig. 1B). The Ca^{2+}-dependent ATPase activity appeared inhibited with increased pre-incubation time when measured in the presence of calcimycin, whereas it appeared enhanced when measured in the absence of the Ca^{2+} ionophore (Fig. 1B).

Table 1: Concentrations of lidocaine for half-maximal inhibition (K_i) of Ca^{2+}-dependent ATPase activity and Ca^{2+} uptake in *temporalis* muscle.

Assay	K_i (mM)
Ca^{2+}-dependent ATPase activity	21.26 ± 1.57 (n = 4)
ATP-dependent Ca^{2+} uptake	26.38 ± 1.22 (n = 4)

The Ki values are reported as mean ± SEM and were significantly different (t=2.57, p=0.042). The values were obtained from four independent experiments performed in duplicate (Figs. 1A and 2A). Data were fitted using a negative slope sigmoid function to estimate K_i values.

Figure 2A shows that lidocaine inhibited the Ca^{2+} uptake in a concentration-dependent manner. The Ca^{2+} uptake decreased upon increasing the lidocaine concentration. The concentration of lidocaine for half-maximal inhibition of Ca^{2+} uptake (K_i) is shown in Table 1.

Figure 2B plots Ca^{2+} uptake as a function of the extravesicular Ca^{2+} concentration. The curve in the absence of lidocaine uncovered a sigmoidal profile corresponding to the calcium activation phenomenon. The presence of lidocaine at 21 and 30 mM in the reaction medium decreased the maximal Ca^{2+} accumulation but did not affect Ca^{2+} affinity.

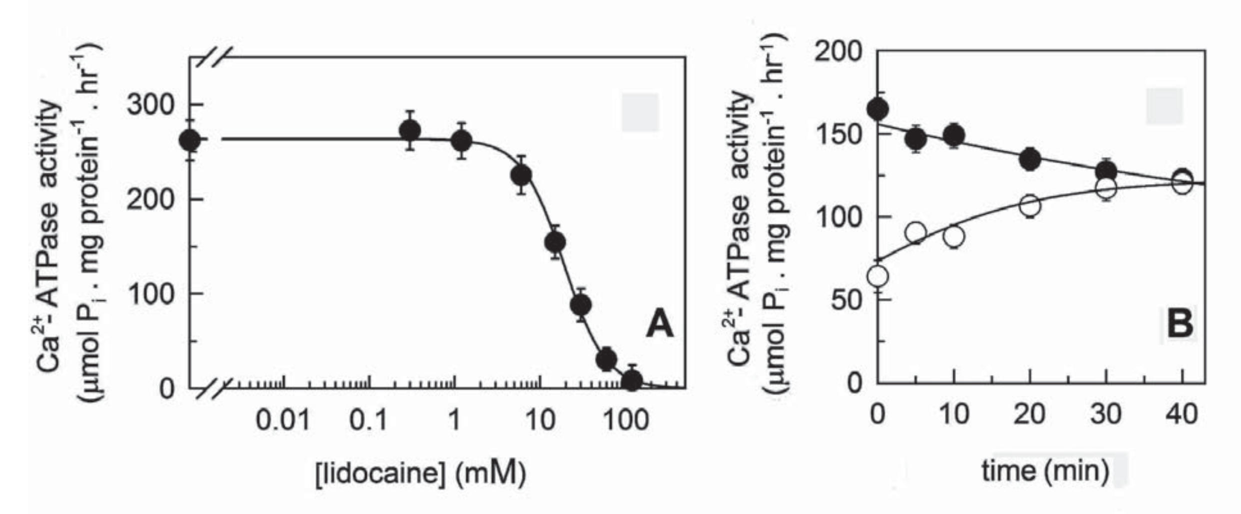

Fig. 1. Ca^{2+}-dependent ATPase activity: (A) Effect of increasing lidocaine concentrations on Ca^{2+}-dependent ATPase activity. Error bars indicate SD; n = 4 (independent experiments performed in duplicate). (B) Effect of lidocaine preincubation time on Ca^{2+}-dependent ATPase activity. (○) without calcimycin. (●) with calcimycin. Error bars indicate SD; n = 4 (independent experiments performed in duplicate).

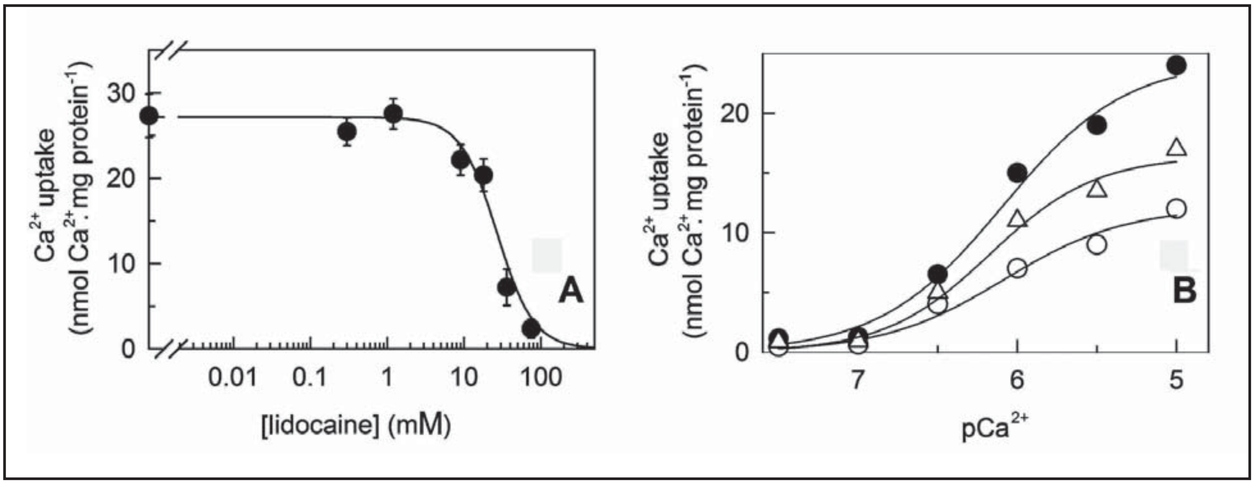

Fig. 2: Effect of lidocaine on the ATP-dependent calcium uptake. (A) Under optimal conditions. Error bars indicate SD; n = 4 (independent experiments performed in duplicate). (B) At different Ca^{2+} concentrations. (●) without lidocaine, (Δ) 21 mM lidoaine, (○) 30 mM lidocaine.

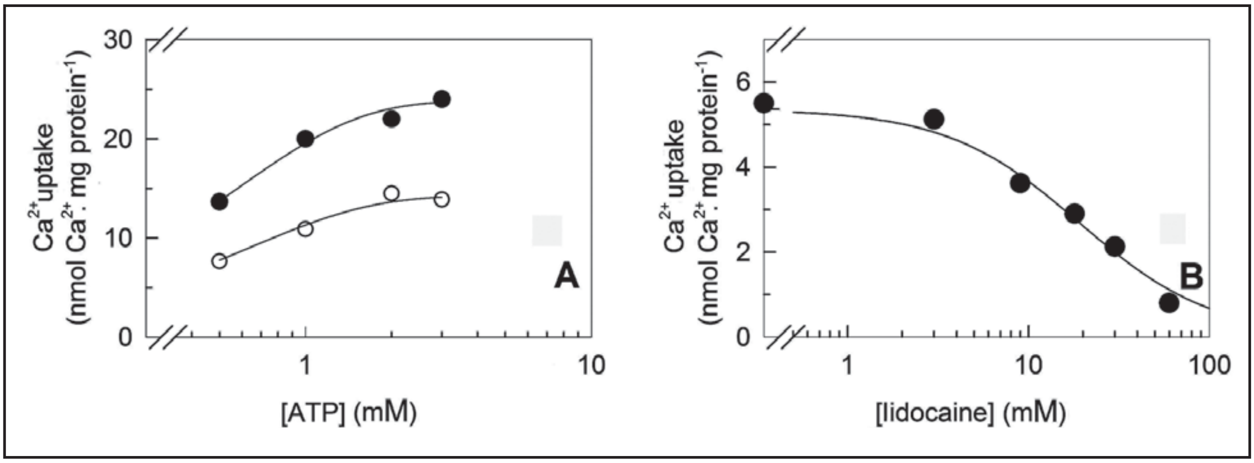

Fig. 3: Effect of lidocaine on the ATP-dependent calcium uptake. (A) At different ATP concentrations. (●) without lidocaine. (○) 30 mM lidocaine. (B) On a single enzyme turnover.

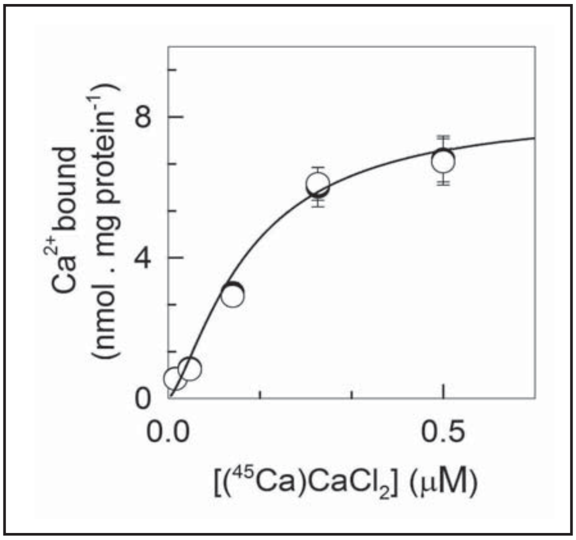

Fig. 4: Effect of lidocaine on Ca^{2+}-binding to the enzyme. (●) without lidocaine. (○) 30 mM lidocaine.

Figure 3A depicts a progressive and saturating increase of Ca^{2+} accumulation as a function of ATP concentration in the absence of lidocaine. Lidocaine 30 mM inhibited the intravesicular Ca^{2+} accumulation. It is also observed that increasing ATP concentrations did not relieve the inhibitory effect of lidocaine. Figure 3B shows that lidocaine inhibited the Ca^{2+} uptake during the first enzymatic cycle in a concentration-dependent manner.

Figure 4 illustrates that Ca^{2+}-binding to the enzyme was not modified by lidocaine.

DISCUSSION

The results reported in this work demonstrate that lidocaine inhibits the Ca^{2+}-dependent ATPase activity in SR membranes from *temporalis* muscle. In addition, the pre-incubation of the SR membranes with lidocaine affects the enzymatic activity. This result demonstrates a dual effect of lidocaine on the Ca^{2+}-dependent ATPase. On the one hand, lidocaine inhibits the optimal Ca^{2+}-dependent ATPase activity measured in the presence of calcimycin. On the other hand, lidocaine increases the SR membrane permeability to Ca^{2+} when the Ca^{2+}-dependent ATPase activity is measured in the absence of the Ca^{2+} ionophore. Furthermore, the increased membrane permeability induced by lidocaine precludes the inhibitory effect of the transmembrane Ca^{2+} gradient increase and the Ca^{2+}-dependent ATPase activity becomes enhanced. This finding points to the ionophoric-like effect of lidocaine.

Whether the Ca^{2+}-dependent ATPase is inhibited or activated depends on the experimental conditions. The results here reported are in line with previous studies in which local anesthetics were able to modify the rate of both Ca^{2+} influx and efflux through either inhibition or activation of the Ca^{2+}-dependent ATPase[12-16]. These effects of the local anesthetics on the SR vesicles suggest that these drugs have multiple sites of action.

The inhibition of the Ca^{2+}-dependent ATPase by lidocaine did not depend on the protein concentration and it was consistent with a moderate octanol/ water partition coefficient[25, 26]. Conversely, for diethyl-estilbestrol and ritodrine, the inhibition of the Ca^{2+}-dependent ATPase decreases upon increasing the protein concentration in the reaction medium[27, 28]. This fact is attributed to drug partitioning into the lipid bilayer.

Lidocaine inhibits Ca^{2+} uptake in SR membranes from *temporalis* muscle. Our result agrees with previous reports in which local anesthetics were found to decrease Ca^{2+} uptake and increase Ca^{2+} efflux in SR membranes from skeletal muscles[11- 16].

The inhibitory effect of lidocaine on Ca^{2+} uptake was reported in fast skeletal muscle[11] and recently in masseter muscle[9]. The results obtained in this work agree with previous reports and suggest that Ca^{2+} has a protective effect on the ATPase at the required lidocaine concentration, i.e. for a given intravesicular Ca^{2+} accumulation level, the extravesicular concentration of the cation becomes higher as lidocaine concentration increases.

Lidocaine concentration that reduces Ca^{2+}-dependent ATPase activity to one half (K_i) was lower than for Ca^{2+} uptake. Regarding this point, it must be considered that Ca^{2+}-dependent ATPase activity was measured in the presence of calcimycin while Ca^{2+} uptake was measured in its absence. We have shown previously[29] that the relative distribution of the intermediate species of the enzymatic cycle depends on the presence or absence of calcimycin. Therefore, the apparent affinity of an inhibitory drug will appear increased or decreased depending on the concentration of the target intermediate species.

We are reporting that K_i value for lidocaine in *temporalis* muscle is lower than in other masticatory muscles[30]. This result indicates a higher affinity of lidocaine for the Ca^{2+}-dependent ATPase in *temporalis* muscle compared to fast skeletal muscle. Previous assumptions on the presence of a different type of Ca^{2+}-dependent ATPase isoform in masticatory muscles[8] could account for this.

The dependence relation between Ca^{2+} uptake and ATP concentration here reported shows that Ca^{2+} uptake increases upon increasing ATP concentration in the presence or absence of lidocaine. However, the maximal Ca^{2+} uptake is lower in the presence of lidocaine. A similar result was observed for Ca^{2+} uptake as a function of Ca^{2+} concentration. Our results demonstrate that the inhibition of lidocaine does not appear to be competitive with respect to Ca^{2+} and ATP.

The study of partial reactions of the Ca^{2+}-dependent ATPase enzymatic cycle allows the action mechanism of different drugs on this enzyme to be elucidated. The experiments where the enzyme was pre-incubated with ^{45}Ca and later ATP, EGTA and lidocaine were added reflect only the transport of calcium bound to the enzyme, and permitted the exploration of steps 1, 2 and 3 of the enzymatic cycle[24]. It could be assumed that step 3 would not be involved in the action of lidocaine, since Ca^{2+} bound to the enzyme is transported even in the presence of lidocaine.

We found that Ca^{2+} transport depends on ATP concentration. However, increasing ATP concentrations does not relieve the inhibitory effect of lidocaine. We cannot discard that interferences in the phosphorylation of the enzyme by ATP would influence Ca^{2+} transport. It is important to remember that Ca^{2+} transport and ATP hydrolysis are regulated by limiting steps of the cycle that could be modified depending on the conditions of the incubation medium. Therefore, the inhibitory effect of lidocaine could affect steps 2 and 4. Several authors have reported that local anesthetics have higher affinity for E_2 and markedly inhibit the phosphorylation of the enzyme by P_i, step 4 of the reverse cycle[14,15,29]. In this work, lidocaine did not affect Ca^{2+}-binding to the Ca^{2+}-dependent ATPase. Since local anesthetics have been reported to be more to likely interact with E_2, the inhibitory effect of lidocaine in step 1 was not expected.

We conclude that the effect of lidocaine on the sarcoplasmic reticulum Ca^{2+}-dependent ATPase from *temporalis* muscle is due to the drug's direct interaction with the enzyme and the increased sarcoplasmic reticulum membrane's permeability to Ca. The inhibition of the Ca^{2+}-dependent ATPase could not fully explain lidocaine myotoxicity, but might induce undesired effects such as sustained contraction of the *temporalis* and masticatory muscles during the treatment of trigeminal neuralgia through infiltrative anesthesia.

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