

EFFECT OF ARTICAININE ON CALCIUM TRANSPORT IN SARCOPLASMIC RETICULUM MEMBRANES ISOLATED FROM MEDIAL PTERYGOID MUSCLE

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ABSTRACT

Local anesthetics used in dentistry have myotoxic effects. Articaine, also known as carticaine, is one of the local anesthetics most widely used in clinical dentistry. The aim of this work was to describe its effect on the sarcoplasmic reticulum Ca-ATPase isolated from medial pterygoid muscle. Ca-ATPase enzymatic activity was determined by a colorimetric method and ATP-dependent calcium uptake with a radioisotopic technique. Articaine inhibited both Ca-ATPase activity and calcium uptake in a concentration-dependent manner. Both inhibitory effects became evident at articaine concentrations lower than those employed in clinical dentistry. Half-maximal inhibitory concentrations ($K_{0.5}$) were $15.1 \pm$

1.8 mM (n = 6) and 25.2 ± 1.6 mM (n = 6) for enzymatic activity and calcium uptake, respectively. Preincubation of sarcoplasmic reticulum membranes with articaine enhanced Ca-ATPase activity in the absence of calcium ionophore, suggesting an ionophoric-like effect of the local anesthetic. We conclude that the inhibitory effect of articaine on the sarcoplasmic reticulum Ca-ATPase isolated from medial pterygoid muscle is due to a direct interaction of the anesthetic with the enzyme and to the increased membrane permeability to calcium induced by this drug.

Keywords: sarcoplasmic reticulum, calcium-transporting ATPases, masticatory muscles, carticaine, anesthetics.

EFFECTO DE LA CARTICAÍNA SOBRE EL TRANSPORTE DE CALCIO EN MEMBRANAS DE RETÍCULO SARCOPLÁSMICO DE MÚSCULO PTERIGOIDEO INTERNO

RESUMEN

Los anestésicos locales de uso odontológico tienen efectos miotóxicos. La carticaína, también conocida como articaine, es uno de los anestésicos locales más usados en la clínica odontológica actual. El objetivo del trabajo fue describir el efecto de la carticaína sobre la Ca-ATPasa del retículo sarcoplásmico aislada del músculo pterigoideo interno. La actividad enzimática de la bomba de calcio se determinó por un método colorimétrico y se utilizó un método radioisotópico a fin de determinar la captación de calcio dependiente de ATP. La carticaína inhibió la actividad enzimática y la captación de calcio en función de su concentración. Ambos efectos se observaron a concentraciones de carticaína menores a las utilizadas en la clínica. Las concentraciones de carticaína necesarias para inhibir la actividad Ca-ATPásica y la captación de calcio a la

mitad de su valor máximo ($K_{0.5}$) fueron 15.1 ± 1.8 mM (n = 6) y 25.2 ± 1.6 mM (n = 6) respectivamente. La preincubación con carticaína de las membranas de retículo sarcoplásmico del músculo pterigoideo interno, en ausencia de ionóforo de calcio, incrementó la actividad de la enzima, evidenciando un efecto ionofórico del anestésico local. Concluimos que el efecto inhibitorio de la carticaína sobre la Ca-ATPasa de retículo sarcoplásmico del músculo pterigoideo interno se debe a la acción directa del anestésico local sobre la enzima y al incremento de la permeabilidad de la membrana del retículo sarcoplásmico al calcio inducido por esta droga.

Palabras clave: retículo sarcoplásmico, ATPasas transportadoras de calcio, músculos masticadores, carticaína, anestésicos.

INTRODUCTION

Depolarization of the sarcolemma causes calcium release from the sarcoplasmic reticulum through a specific calcium channel located on the terminal cisternae and the rise of myoplasmic calcium triggers muscle contraction¹. The sarcoplasmic reticulum Ca-ATPase is a membrane bound protein which pumps calcium towards the reticulum lumen leading to muscle relaxation². It keeps the myoplasmic calcium concentration at a low level around 100 nM¹.

The medial pterygoid is a masticatory muscle heterogeneously composed of slow and fast fibers, which has a key role in mastication³. It has several actions such as elevation, protraction and displacement of the mandible. It delimitates the pterygomandibular space, where dental local anesthetics are injected to obtain effective inferior alveolar nerve block⁴. The functional characteristics of the Ca-ATPase from medial pterygoid muscle have been reported by Sánchez et al.⁵.

Local anesthetics have myotoxic effects. They can cause ultrastructural damage^{6,7} and promote histochemical changes⁸ in skeletal muscle. Dental local anesthetics have been reported to inhibit the sarcoplasmic reticulum Ca-ATPase isolated from fast skeletal muscles⁹⁻¹². Lidocaine and bupivacaine have been recently reported to inhibit Ca-ATPase activity and calcium transport in sarcoplasmic reticulum membranes isolated from masticatory muscles, such as masseter, medial pterygoid¹³ and temporalis muscles¹⁴.

Articaine, also known as carticaine, is a local anesthetic frequently used in clinical dentistry^{15,16}. It has been reported to inhibit Ca-ATPase activity and calcium transport in sarcoplasmic reticulum membranes from fast skeletal muscles^{11,12}. Its effect on Ca-ATPase isolated from masticatory muscles has not been reported yet. The likely link between the sarcoplasmic reticulum Ca-ATPase from medial pterygoid muscle and myotoxicity of articaine in masticatory muscles motivated this study.

The aim of this work was to describe the effect of articaine on the sarcoplasmic reticulum Ca-ATPase isolated from medial pterygoid muscle. We tested the hypothesis that articaine increases myoplasmic calcium level by inhibiting Ca-ATPase activity and calcium transport at a concentration similar to or lower than that employed in clinical dentistry.

MATERIALS AND METHODS

Membrane Preparation

Medial pterygoid muscles were sampled from adult New Zealand rabbits (6 months old, males, 2 kg) for the isolation of sarcoplasmic reticulum membranes according to Champeil et al.¹⁷. Membranes were obtained as sealed vesicles with ATP-dependent calcium accumulation ability and calcium-dependent ATPase activity. Protein concentration was determined by the method of Lowry et al.¹⁸. This work was carried out in accordance with the guidelines laid down by the National Institute of Health (USA) and in accordance with local laws and regulations regarding the care and use of animals for experimental procedures.

Calcium-dependent ATPase activity

Sarcoplasmic reticulum membranes (0.1mg/ ml) were incubated at 37°C for 2 min in 50 mM MOPS-Tris buffer (pH 7.2), 10 μ M calcium ionophore A23187, 3 mM ATP, 100 mM KCl, 3 mM MgCl₂,

0.1 mM CaCl₂, 0.1 mM EGTA and articaine at various concentrations. Reactions were stopped with 5% trichloroacetic acid (final concentration). After separation of denatured proteins, inorganic phosphate (P_i) was measured with the method of Baginski et al.¹⁹, and taken as an index of Ca-ATPase enzymatic activity. When indicated, prior to incubations, membranes (0.5 mg protein/ ml) were preincubated for 20 min with articaine at different concentrations and 50 mM MOPS-Tris buffer (pH 7.2) or with 15 mM articaine at different preincubation times. Later, the media were diluted 1:5 in solutions as described above with or without articaine. Blanks without sarcoplasmic reticulum membranes were run in parallel and subtracted from the experimental values.

ATP-dependent calcium uptake

Sarcoplasmic reticulum membranes (0.1mg/ ml) were incubated at 37°C for 30 sec in 3 mM ATP, 100 mM KCl, 3 mM MgCl₂, 0.1 mM (⁴⁵Ca)CaCl₂ (450 cpm/ nmol), 0.1 mM EGTA, 50 mM MOPS-Tris buffer (pH 7.2) and articaine at different concentrations. Reactions were stopped by filtration (Millipore filters, 0.45 μ m average pore size, Bedford, MA, USA). Filters were immediately washed with cold 3 mM LaCl₃ and retained radioactivity measured in a liquid scintillation counter. Blanks without ATP were run in parallel and subtracted from the experimental values.

Chemicals and radioisotopes

Disodium ATP (adenosine triphosphate), calcium ionophore A23187, bovine seroalbumin, MOPS (3-[n-morpholino]propanesulfonic acid) and Tris (Tris[hydroxymethyl]aminomethane) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade. (⁴⁵Ca)CaCl₂ was from New England Nuclear (E.I. Dupont de Nemours, Boston, MA, USA). Articaine chlorhydrate was a kind gift from Septodont (France).

Data analysis

Mean values \pm SEM of the results are given. Dose-response curves were fitted using GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego CA, USA). Differences in half-maximal inhibition concentration (K_i) of articaine for Ca-ATPase activity and calcium uptake were tested for significance by Student's t test (p < 0.05).

RESULTS

Our experiments were devised to examine the effect of articaine on the catalytic and transport activities of the Ca-ATPase isolated from medial pterygoid muscle. It is well known that calcium accumulation inside SR vesicles inhibits Ca-ATPase activity and

that its full assessment requires a calcium permeable membrane, usually achieved with calcium ionophore. This study was therefore conducted by measuring the rate of ATP hydrolysis using a preparation of sarcoplasmic reticulum vesicles rendered leaky by addition of the calcium ionophore A23187.

Experimental conditions included a buffered medium at pH 7.2, the presence of Ca^{2+} , Mg^{2+} , K^{+} , and ATP as a phosphate-donor substrate. The maximal rate of Ca-ATPase activity measured under these conditions was $250 \mu\text{mol P}_i$ per mg protein/h. Articaine inhibited Ca-ATPase activity in a concentration-dependent manner. A plot of enzyme activity vs concentration of articaine (Fig. 1) provided a half-maximal inhibitory concentration (K_i) of $15.1 \pm 1.8 \text{ mM}$ ($n = 6$). It is also noteworthy that articaine showed practically no inhibitory effect at concentrations lower than 10 mM, while the maximal inhibitory effect of the anesthetic was obtained at a concentration of about 100 mM. The effect of articaine was not dependent on protein concentration, as varying the aliquot of sarcoplasmic reticulum membrane added to the assay media from 0.05 to 0.2 mg/ml had no effect on Ca-ATPase activity (Fig. 1).

To test the likely membrane-permeabilizing effect of articaine, we determined Ca-ATPase activity in the absence of the calcium ionophore after preincubation of the sarcoplasmic reticulum membranes with articaine at different concentrations. Ca-ATPase activity first increased upon the increase in articaine concentration up to approximately 10 mM and it was inhibited at higher concentrations (Fig. 2). This result suggested the ionophoric-like effect of articaine on sarcoplasmic reticulum medial pterygoid membranes. To obtain additional information regarding this effect, we determined Ca-ATPase activity as a function of articaine preincubation time (Fig. 3). Based on the above results, we selected a concentration (15 mM) that enhances calcium permeability without

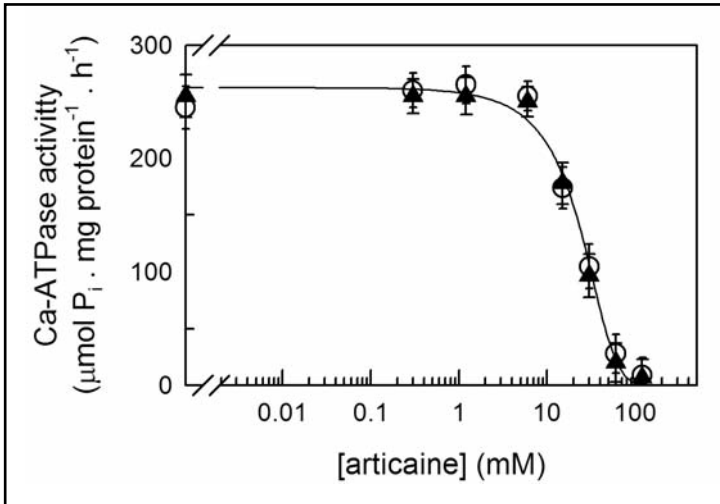


Fig. 1: Inhibitory effect of articaine on sarcoplasmic reticulum Ca-ATPase activity. Sarcoplasmic reticulum membranes (\circ) 0.05 mg/ml and (\blacktriangle) 0.2 mg/ml were incubated with varying concentrations of articaine as indicated and Ca-ATPase activity measured spectrophotometrically as described in Methods. Results are expressed as mean $\mu\text{mol P}_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1} \pm \text{SEM}$ ($n = 6$) and data were fitted with a single sigmoidal curve with $K_i = 15.1 \text{ mM}$.

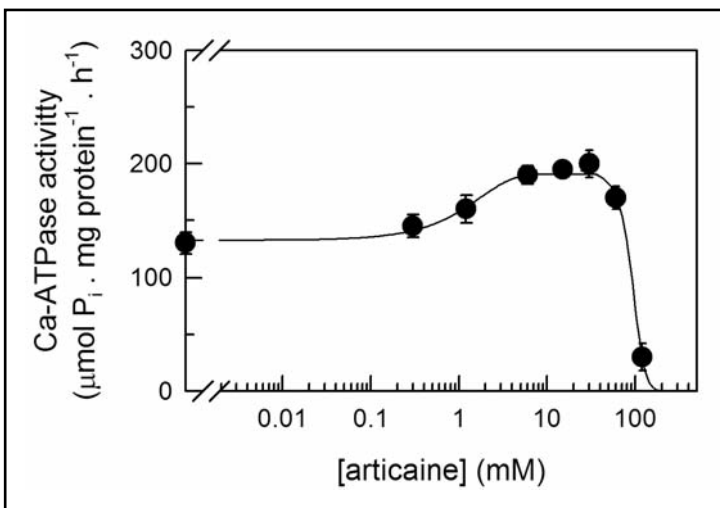


Fig. 2: Concentration-dependent effect of articaine preincubation on Ca-ATPase activity. Sarcoplasmic reticulum membranes (0.5 mg/ml) were preincubated for 20 min with varying articaine concentrations as indicated, the reaction medium was then diluted 1:5 with reaction buffer excluding calcium ionophore and Ca-ATPase activity measured spectrophotometrically as described in Methods. Results are expressed as mean $\mu\text{mol P}_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1} \pm \text{SEM}$ ($n = 6$).

producing full Ca-ATPase activity inhibition. Enzymatic activity varied according to the time the sarcoplasmic reticulum membrane preparation was preincubated with the local anesthetic. It was inhibited or enhanced depending on the presence or absence of calcimycin, respectively. Both effects of articaine depended on the preincubation time.

To study the effect of articaine on calcium transport in sarcoplasmic reticulum membranes isolated from medial pterygoid muscles, we determined the calcium uptake as a function of the local anesthetic concentration (Fig. 4). Maximal calcium uptake obtained in the absence of the anesthetic was 28 nmol Ca/mg protein. Articaine inhibited ATP-dependent calcium uptake in a concentration-dependent manner within a concentration range up to approximately 100 mM and half-maximal inhibitory concentration (K_i) of 25.2 ± 1.6 mM ($n = 6$). This K_i value was significantly higher ($t_{(10)} = 4.56$, $p = 0.001$) than that obtained for Ca-ATPase activity (15.1 ± 1.8 mM).

DISCUSSION

This is the first study investigating the effect of articaine on sarcoplasmic reticulum Ca-ATPase isolated from a masticatory muscle. Our results show that articaine inhibits Ca-ATPase activity of the medial pterygoid sarcoplasmic reticulum membrane. The molecular structure of articaine has a thiophene aromatic ring and recalls that of other Ca-ATPase inhibitors. Earlier studies indicate that hydrophobic compounds having aromatic rings act as inhibitors of this enzyme. This includes the antipsychotic drug trifluoperazine^{20,21}, the β -adrenergic agonist ritodrine²², the neuroleptic drug haloperidol²³ and the synthetic nonsteroidal estrogen diethylstilbestrol²⁴. In some cases, such as diethylstilbestrol, the extent of enzyme inhibition decreased as a function of membrane protein concentration. This has been attributed to drug partitioning

into the lipid bilayer. Alternatively, a similar dependence on membrane protein concentration can be observed in the presence of specific tight-binding inhibitors such as thapsigargin²⁵, although the mechanism of action is different. The inhibitory effect of articaine did not depend on enzyme protein concen-

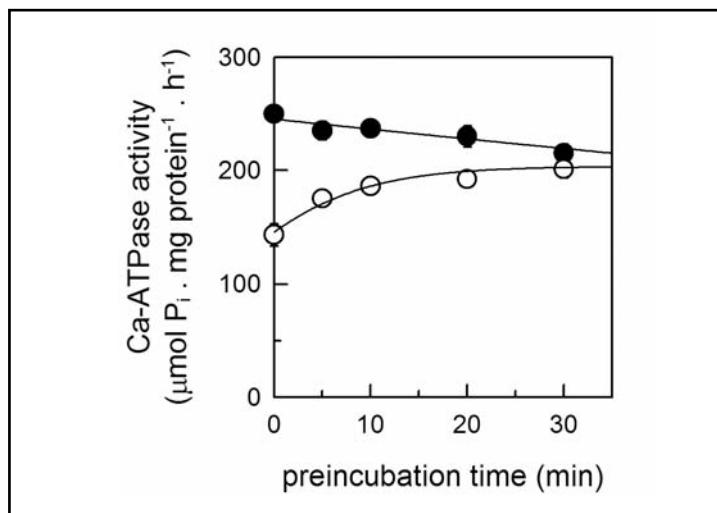


Fig. 3: Time-dependent effect of articaine preincubation on Ca-ATPase activity. Sarcoplasmic reticulum membranes (0.5 mg/ml) were preincubated during the indicated times with 15 mM articaine in the presence (●) or the absence (○) of calcium ionophore and Ca-ATPase activity measured spectrophotometrically as described in Methods. Results are expressed as mean $\mu\text{mol P}_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1} \pm \text{SEM}$ ($n = 6$).

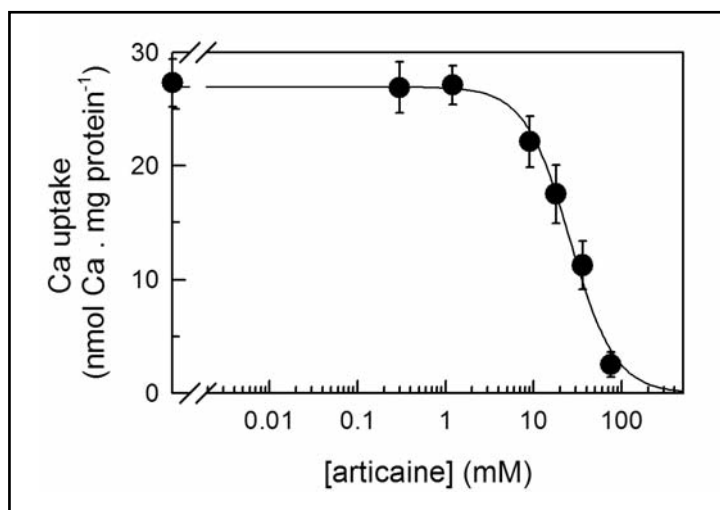


Fig. 4: Inhibitory effect of articaine on calcium uptake by sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum membranes (0.1 mg/ml) were incubated with varying concentrations of articaine as indicated and Ca uptake determined as ⁴⁵Ca incorporation as described in Methods. Results are expressed as mean nmol Ca · mg protein⁻¹ \pm SEM ($n = 6$) and data were fitted with a single sigmoidal curve with $K_i = 25.2$ mM.

tration; therefore we can rule out a membrane partition mechanism or a thapsigargin-like effect.

Articaine has been reported to inhibit Ca-ATPase activity and calcium transport in sarcoplasmic reticulum membranes isolated from other skeletal muscles^{11,12}. Such inhibitory effects have been described at articaine concentrations higher than those here reported. This indicates a higher affinity of the Ca-ATPase isolated from medial pterygoid muscle for articaine. It is noteworthy that similar results have been previously reported for lidocaine and bupivacaine¹³. Previous assumptions on the presence of a different type of Ca-ATPase isoform or the mixture of two or more isoforms in masticatory muscles⁵ could explain this fact. An interesting feature of the inhibitory effect of articaine on Ca-ATPase activity was its modulation by preincubation of the enzyme with articaine at different experimental times. This demonstrated the dual effect of articaine. On one hand, articaine inhibited the optimal Ca-ATPase activity measured in the presence of the calcium ionophore but, on the other hand, in the absence of the calcium ionophore, articaine increased membrane permeability, thus enhancing Ca-ATPase activity by preventing the inhibitory effect of intravesicular calcium accumulation.

Articaine also inhibited calcium transport in sarcoplasmic reticulum medial pterygoid membranes by inhibition of the Ca-ATPase. Similar results have been reported for membranes isolated from other skeletal muscles^{11,12}, although the K_i value here reported is lower. Again, this fact indicates a higher affinity of the Ca-ATPase isolated from medial pterygoid muscle for the local anesthetic articaine. The inhibitory effect of articaine on calcium uptake by Ca-ATPase is important because it prevents the active calcium accumulation inside the reticulum lumen, thus increasing the myoplasmic calcium concentration. Increased myoplasmic calcium favors muscle contraction and prevents its relaxation. As a result, the likely clinical picture is

sustained muscle contraction. Another interesting point to be discussed is the higher K_i value reported for calcium uptake compared to that obtained for Ca-ATPase activity. It should be noted that Ca-ATPase activity was measured in the presence of a calcium ionophore while calcium uptake was measured in its absence. As a consequence, the relative distributions of the intermediate species of the Ca-ATPase enzymatic cycle during the steady state of the reactions were different under both conditions¹². Because Ca-ATPase undergoes conformational changes throughout its cycle, different affinities of any inhibitory drug could be expected for each of the different enzymatic conformations; thus, any change in the relative distribution of enzyme conformers must affect the K_i value of the inhibitory drug.

Regarding the clinical significance of the findings reported in this study, it is interesting to compare obtained K_i values for articaine with the drug's available concentration in dental cartridges. This comparison reveals that articaine inhibitory effects on Ca-ATPase activity and calcium transport occur at concentrations lower than the commercially available 4% articaine formulation (125 mM). This indicates that myotoxic effects are likely to be observed after the diffusion of articaine into muscle fibers. Such diffusion, for instance, could occur following inadvertent injection of the local anesthetic into the medial pterygoid muscle fibers during the sensitive blockade of the inferior alveolar nerve.

We conclude that the inhibitory effect of articaine on sarcoplasmic reticulum Ca-ATPase isolated from medial pterygoid muscle is due to the direct interaction of the anesthetic with the enzyme and the increased membrane permeability to calcium induced by this drug. Although inhibition of Ca-ATPase activity and of calcium transport could only partly explain the myotoxic effects of articaine, this study provides useful evidence to the background of articaine myotoxicity.

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