Hokkaido J. Dent. Sci., 43 : 57−62, 2022.

ORIGINAL

Reversible inhibition of Ca^{2+}- or Mg^{2+}-dependent ATPase activity in the rat brain by local anesthetics

Rie Iwamoto, Kuniaki Suzuki, Yuri Hase, Makiko Shibuya,
Yukifumi Kimura and Toshiaki Fujisawa

ABSTRACT :

Background: Local anesthetics can easily pass through the blood-brain barrier and may cause adverse effects in the brain; however, the direct influence of these effects on the central nervous system remains to be clarified. ATPases activated by Ca^{2+} (Ca-ATPase) or Mg^{2+} (Mg-ATPase), which are different from the plasma membrane (PMCA) or the sarco-endoplasmic reticulum (SERCA) Ca, Mg-ATPases, exist in the brain. There are some reports on the effect of local anesthetics on PMCA and SERCA, but few have described the effects on Ca- or Mg-ATPase. The aim of our study was to describe the effect of local anesthetics on these ATPases.

Methods: We isolated plasma membrane (PII) and microsomal (PIII) fractions from rat brain homogenates and examined the effects of local anesthetics, procaine, tetracaine, lidocaine, prilocaine, bupivacaine, and dibucaine on Ca- or Mg-ATPase activities at pH 7.4 or 9.5.

Results: The Ca- and Mg-ATPase activities of the PII fraction were inhibited in a dose-dependent manner by all local anesthetics at pH 9.5, which is the range of clinical use. Tetracaine and dibucaine, which are clinically strong anesthetics, showed strong inhibitory effects on ATPase activity. The inhibition of activity by lidocaine, tetracaine, and dibucaine was recovered after their concentrations were diluted, suggesting that their inhibitory actions were reversible.

Conclusion: These results suggest that local anesthetics at concentrations available for dental use, reversibly inhibit PII Ca- or Mg-ATPase activities in the rat brain.

Key Words : Anesthetics local; Brain; Ca^{2+}, Mg^{2+}-ATPase; Dibucaine; Lidocaine; Tetracaine.

Introduction

Local anesthetics bind to the Na^+ channel and inhibit Na^+ entry into nerve cells[1, 2]. However, the action of local anesthetics is not specific to the sensory nerves; as they affect all membranes, including the motor and autonomic nerves. These actions may lead to adverse effects, which are difficult to explain by the inhibition of the Na^+ channel[3, 4]. All local anesthetics can easily pass through the blood-brain barrier. The entry of local anesthetics into the brain owing to an increase in blood concentration due to overdosage may cause central action[3−7]. Excitation of the central nervous system may be caused by blocking the inhibitory system of the cerebral cortex, or by stimulating the release of excitable neurotransmitters such as glutamate; however, the details regarding the effect of local anesthetics remain to be clarified.

Several ATPases are activated in the brain by Ca^{2+} or Mg^{2+}, which may be related to ion transport, signal transduction, or energy conversion[8−12]. A well-known ATPase is the Ca, Mg-ATPase in the plasma membrane (PMCA) and sarco-endoplasmic reticulum (SERCA)[8−10]. There are reports regarding the effects of local anesthetics on PMCA in the brain[13, 14]. However, there are other Ca^{2+}- or Mg^{2+}-dependent ATPases (Ca- or Mg-ATPases) that differ from either PMCA or SERCA, and their functions are not clear[11, 12]. To the best of our knowledge, there are no reports on the effects of local anesthetics on Ca- or Mg-ATPases. We considered the possibility that the inhibition of Ca- or Mg-ATPase is

Department of Dental Anesthesiology, Faculty of Dental Medicine and Graduate School of Dental Medicine, Hokkaido University, Sapporo, Hokkaido 060-8586, Japan

involved in the adverse effects of local anesthetics; therefore, we studied the effect of local anesthetics on Ca- or Mg-ATPase activity.

Materials and methods

Isolation of plasma membrane (PII) and microsomal (PIII) fractions from the rat brain

Fifty male Slc:Wistar rats aged 5 weeks and weighing 150-160 g (Sankyo Labo Service Corporation, Inc., Sapporo, Japan) were used. The rats were treated under an institutionally approved protocol for animal research at the Hokkaido University (No.16-0026). The rats were housed in an animal facility under controlled temperature and humidity conditions and a 12-h light/dark cycle with free access to food and water. They were exposed to carbon dioxide in their home cage and euthanized by slow displacement of the air in the cage. We verified the death of the rats and immediately removed the whole brains from the rats for further analyses.

We isolated PII and PIII fractions from the rat brain according to the method described by Pottorf[15]. First, the whole rat brain was cut and blended after removal of connective tissue to obtain the homogenate. PI was obtained by precipitation from the homogenate, which was separated by centrifugation at 2,000 $\times g$ for 15 min. Then, PII was separated from the supernatant of PI by centrifugation at 20,000 $\times g$ for 30 min, and PIII was separated from the supernatant of PII by centrifugation at 100,000 $\times g$ for 75 min.

Measurement of Ca- or Mg-ATPase activity of PII or PIII fractions and the effects of local anesthetics on ATPase activity

We determined Ca- or Mg- ATPase activity at pH 7.4 or 9.5. The 250 µl reaction mixture comprised 14-15 µg of the PII or PIII fraction, a final concentration of 2 mM $CaCl_2$ (Ca-ATPase) or 2 mM $MgCl_2$ (Mg-ATPase), 25 mM sucrose, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 50 mM Tris[hydroxymethyl]aminomethane (Tris)-HCl buffer at pH 7.4 or 9.5, with (Mg-ATPase) or without (Ca-ATPase) 0.7 mM sodium azide. After 10 min of preincubation at 37 ℃, the reaction was started by the addition of 50 µl of 30 mM ATP-Tris and allowed to proceed for 60 min at 37 °C. Reactions were stopped by the addition of 12% sodium dodecyl sulfate. Inorganic phosphate formed by the hydrolysis of ATP was detected according to Chifflet's method[16]. The developed color was read spectrophotometrically at 850 nm using a Hitachi U- 2000 spectrophotometer (Hitachi, Tokyo, Japan).

To test the effects of local anesthetics on ATPase activity, final concentrations (0.31, 0.63, 1.25, 2.5, 5, and 10 mM) of procaine, tetracaine, lidocaine, prilocaine, bupivacaine, or dibucaine, were added to the reaction mixture, as shown in Figs. 1 and 2. As 10 mM tetracaine inhibited the activities of PII Ca-ATPase by more than 80% and that of PII Mg-ATPase by 90% in preliminary experiments, we tested the local anesthetics at concentrations of 0 to 10 mM.

Reversibility of inhibited Ca- or Mg-ATPase activities after dilution of local anesthetic concentrations

To examine whether inhibition by local anesthetics was reversible, we performed the following tests. First, a reaction mixture containing 22-24 µg of PII fraction, 25 mM sucrose, 0.1 mM EDTA, 50 mM Tris-HCl at pH 9.5, 2 mM $CaCl_2$ (Ca-ATPase) or 2 mM $MgCl_2$ (Mg-ATPase), with (Mg-ATPase) or without (Ca-ATPase) 0.7 mM sodium azide was prepared, along with (dilution group) or without (non-dilution group) tetracaine, dibucaine, or lidocaine, at concentrations that inhibited Ca- or Mg-ATPase activity. The prepared mixtures were incubated for 30 min at 20-25 °C. Next, 190 µl of a solution containing 25 mM sucrose, 0.1 mM EDTA, 50 mM Tris-HCl at pH 9.5, and 2 mM $CaCl_2$ or $MgCl_2$ and 30 µl of tetracaine, dibucaine, or lidocaine to give the final concentrations shown in the x-axes of Figs. 3 and 4 was prepared and added to 30 µl of the above reaction mixture for the dilution or non-dilution groups. The final concentrations of tetracaine, dibucaine, or lidocaine in the comparable dilution or non-dilution groups were the same. After 10 min of preincubation at 37 °C, the ATPase reaction was started by the addition of 50 µl of 30 mM ATP-Tris and allowed to proceed for 60 min at 37 °C. Inorganic phosphate production by ATP hydrolysis was measured as described above.

Drugs and chemicals

Local anesthetics (procaine, tetracaine, lidocaine, prilocaine, bupivacaine, and dibucaine) and ATP were purchased from Sigma (St. Louis, MO, USA). All other drugs and chemicals used were of analytical grade.

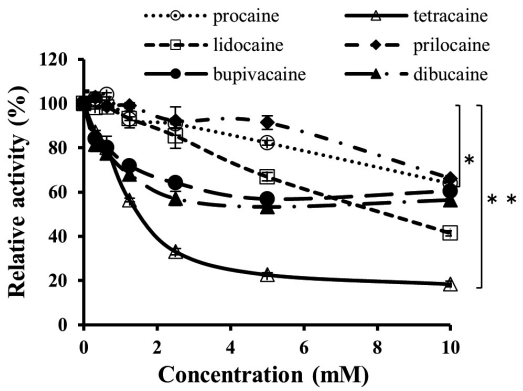

Fig. 1 Inhibition of PII Ca- ATPase activity by local anesthetics (pH 9.5)

Local anesthetics (procaine, tetracaine, lidocaine, prilocaine, bupivacaine and dibucaine) were tested at final concentrations of 0.31, 0.63, 1.25, 2.5, 5, and 10 mM. Each value represents mean ± standard deviation (n = 3). *(P = 1.55E-02), **(P = 1.09E-04) compared to the relative activity (%) without local anesthetics.

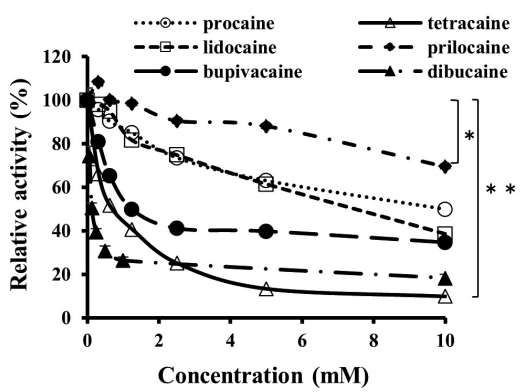

Fig. 2 Inhibition of PII Mg-ATPase activity by local anesthetics (pH 9.5)

Local anesthetics were tested at final concentrations of 0.06, 0.13, 0.25, 0.31, 0.5, 0.63, 1.25, 2.5, 5, and 10 mM. Each value represents mean ± standard deviation (n = 3). *(P = 7.24E-03), **(P = 7.17E-07) compared to the relative activity (%) without local anesthetics.

Statistical procedure

All measurements were performed in triplicate for each concentration, and the means and standard deviations are shown in each graph. When the error bar is not shown, the error bar size is smaller than the size of the symbol. Statistical analysis of the data was performed using the unpaired Student's *t*-test. P-values for each bracket * and ** were shown in the figure legends of Fig. 1 and 2. Concentrations that resulted in half maximal inhibition (IC_{50}) were calculated using the Hill plot.

Results

Inhibition of PII Ca-ATPase activity by local anesthetics at pH 9.5

We tested the inhibition of PII and PIII Ca-ATPase activity by local anesthetics at pH 7.4. Common inhibition of activity was not observed with any of the local anesthetics tested at this pH (data not shown).

With all the local anesthetics tested, PII Ca-ATPase activity decreased in a concentration-dependent manner at pH 9.5 (Fig. 1). Inhibition by tetracaine was the strongest (IC_{50}: 1.1 mM) (Table 1).

Inhibition of PII Mg-ATPase activity by local anesthetics at pH 9.5

We tested the inhibition of PII and PIII Mg-ATPase activity by local anesthetics at pH 7.4; however, no common inhibition of activity by any of the tested local anesthetics was observed (data not shown).

PII Mg-ATPase activity decreased in a concentration-dependent manner with all the local anesthetics tested at pH 9.5 (Fig. 2). Inhibition by dibucaine was the strongest (IC_{50}: 0.3 mM), followed by tetracaine (IC_{50}: 0.7 mM) (Table 1).

Table 1. IC_{50} values for each anesthetic for PII Ca-ATPase and Mg-ATPase activity (pH 9.5).

anesthetics	IC_{50} (mM)	
	PII Ca-ATPase (pH 9.5)	PII Mg-ATPase (pH 9.5)
procaine	21.3	9.5
tetracaine	1.1	0.7
lidocaine	7.5	7.0
prilocaine	15.4	16.5
bupivacaine	n.d.	1.4
dibucaine	n.d.	0.3

IC_{50} values were calculated using the Hill plot.

n.d.: When maximum inhibition was less than 50 %, the IC_{50} values were not determined.

Reversibility of PII Ca- or Mg-ATPase activities inhibited by tetracaine

We examined the reversibility of ATPase activity inhibited by local anesthetics, as described in the Materials and methods section. Fig. 3 shows the reversibility of the PII Ca-ATPase activity that was inhibited by tetracaine. The activities after dilution (◆) and non-dilution (□) are shown, and the final concentrations tested are shown on the x-axis. The inhibition of activity in the presence of 5 mM tetracaine (◆) recovered after dilution, to a level similar to that obtained in the presence of the same final concentrations without dilution. The inhibitory activity obtained for tetracaine (Fig. 1, △) is also shown for comparison.

Fig. 4 shows similar results regarding the reversibility of PII Mg-ATPase after dilution (◆), non-dilution (□), and the initial data (Fig. 2, △) obtained. The inhibition of Mg-ATPase activity in the presence of 2.5 mM tetracaine (◆) recovered after dilution, to a level similar to that obtained in the presence of the same final concentrations without dilution.

Similar results were obtained for lidocaine and dibucaine regarding the reversal of PII Ca- or Mg-ATPase activity inhibition after dilution of the anesthetic concentration (data not shown).

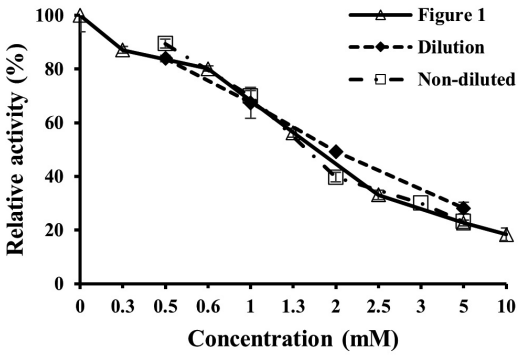

Fig. 3 Reversibility of PII Ca- ATPase activities inhibited by tetracaine (pH 9.5)

Tetracaine (5 mM) in dilution or non-diluted was tested and compared with the inhibitory activity observed in Fig. 1. Each value represents mean ± standard deviation (n = 3). The r^2 values were 0.982 for Figure 1, and 0.997 for the Dilution and 0.968 for Non-diluted samples.

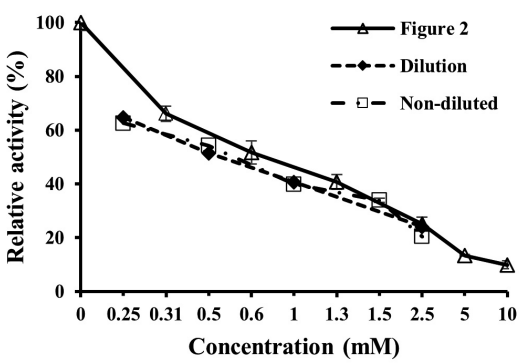

Fig. 4 Reversibility of PII Mg-ATPase activities inhibited by tetracaine (pH 9.5)

Tetracaine (2.5 mM) in dilution or non-diluted was tested and compared with the inhibitory activity observed in Fig. 2. Each value represents mean ± standard deviation (n = 3). The r^2 values were 0.968 for Figure 2, and 0.998 for the Dilution and 0.967 for Non-diluted samples.

Discussion

Inhibition of plasma membrane Ca-ATPase activity by local anesthetics in the brain

Ca^{2+} is important for signal transduction and the activation of enzymes in the brain. Therefore, it is important to maintain the concentration gradient of Ca^{2+} across the plasma membrane to maintain the function of Ca^{2+}-dependent systems[10, 11]. Many Ca^{2+}-dependent ATPases are present in the brain[8–12]. Among them, the most studied Ca^{2+}-dependent ATPases are PMCA and SERCA[8–10]; some studies have reported that local anesthetics inhibit the ATPase activities of PMCA in the brain[13, 14] and SERCA in the skeletal muscle[17–20]. In contrast, there have been many reports of Ca^{2+}-dependent ATPases, which differ from PMCA and SERCA, in various tissues, including the brain. However, these Ca-ATPases have not been extensively studied in the past 30 years. There are no reports regarding the effects of local anesthetics on these Ca-ATPases. We tested the hypothesis that local anesthetics inhibit these Ca-ATPases.

The Ca-ATPase activity of the PII fraction at pH 9.5 was inhibited in a dose-dependent manner by local anesthetics, especially by tetracaine, which has strong local anesthetic action and adverse effects (Fig. 1). In the case of lidocaine, which is used frequently, 10 mM lidocaine decreased the activity to 41%. The molar concentration of 2% lidocaine is equivalent to 74 mM, and lidocaine easily passes through the blood-brain barrier. If the blood concentration increases due to overdosage, lidocaine may inhibit Ca-ATPase activity to some extent in the brain and cause central action[3–5].

Local anesthetics with strong action showed strong inhibition of Ca-ATPase; such inhibition may be a risk factor for inducing adverse effects. The mechanism of local anesthetics is established as inhibition of Na^+ channel

function, so reactions other than Na^+ channel reactions are side effects. Local anesthetics have many adverse effects on the central nervous system; however, the mechanism is not clear[3–7]. Therefore, we speculated that the inhibition of Ca-ATPases by local anesthetics may be related to adverse effects.

Garcia-Martin and Gutiérrez-Merino showed that local anesthetics inhibit the PMCA in rat brain synaptosomes at concentrations close to their respective pharmacological doses[13, 14]. The Ca-ATPase studied in this article is different from PMCA, because PMCA is activated by submicromolar Ca^{2+} concentrations; however, Ca-ATPase in this study is activated by millimolar Ca^{2+} concentrations[10, 11]. This suggests that this Ca-ATPase can transport higher amounts of Ca^{2+} compared with PMCA and contributes to maintaining the Ca^{2+} gradient and Ca^{2+}-dependent functions. When local anesthetics inhibit this ATPase, the Ca^{2+} gradient necessary for Ca^{2+}-dependent systems in the brain will be disturbed and the functions of the systems will be inhibited. The function of these Ca-ATPases remains to be clarified[11, 12], and further study is necessary to fully understand the physiological effects of inhibition by local anesthetics.

Inhibition of plasma membrane Mg-ATPase activity in the brain by local anesthetics

The Mg-ATPase activity of the PII fraction at pH 9.5 was inhibited in a dose-dependent manner by local anesthetics, especially by dibucaine and tetracaine, which have strong local anesthetic action and adverse effects (Fig. 2). Lidocaine (10 mM) inhibited PII Mg-ATPase activity by 41%. Local anesthetics may inhibit Mg-ATPase activity in the brain, and this inhibition may be a risk factor for adverse effects, similar to the inhibition of Ca-ATPase described above.

There are reports of Mg^{2+}-dependent ATPases in the brain[11, 21]. Some of these are known as F-ATPase or ethacrynic acid-dependent Mg-ATPase, one of which is basal Mg-ATPase, whose character and function are not clear. There are no reports on the effects of local anesthetics on basal Mg-ATPase. We showed the inhibition of these Mg-ATPases by local anesthetics. If local anesthetics inhibit this Mg-ATPase in the brain, the ATPase functions will be inhibited. Further investigation is necessary to reveal the function of basal Mg-ATPases to understand the physiological effects of inhibition by local anesthetics.

Reversibility of the activity inhibited by local anesthetics

Local anesthesia is reversible because inhibition of the Na^+ channel is reversible. The adverse effects of local anesthetics on the central nervous system are also usually reversible. We assume that if the inhibition of Ca- or Mg-ATPase by local anesthetics is related to adverse effects, the reversal of the inhibition should allow recovery from adverse effects on the central nervous system.

We tested the reversibility of ATPase inhibition by local anesthetics by diluting the anesthetic concentrations[22]. If the inhibition is reversible, the inhibited activity will recover on dilution. If the inhibition is irreversible, such as through the formation of covalent bonds, the activity will not be recovered by dilution.

As shown in Figs. 3 and 4, the activities of both Ca- and Mg-ATPase, inhibited by tetracaine, recovered after the dilution of tetracaine concentrations, suggesting that inhibition by tetracaine is reversible. Inhibition by lidocaine and dibucaine also recovered after dilution (data not shown).

These results suggest that the activities of Ca- and Mg-ATPase can be reversibly inhibited by local anesthetics in the rat brain. Similar results have been reported for Na, K-ATPase, which is essential for nerve function[22]. These inhibitions may be related to the adverse effects of local anesthetics in the brain and should be investigated further.

Acknowledgements:

We would like to thank Editage (www.editage.com) for English language editing.

Competing interests:

The authors declare that they have no competing interests.

References

1) Butterworth II JF, Strichartz GR : Molecular mechanisms of local anesthesia: A review. Anesthesiology 72 : 711-734, 1990.
2) Ragsdale DS, McPhee JC, Scheuer T, Catterall WA : Molecular determinants of state-dependent block of Na^+ channels by local anesthetics. Science 265 : 1724-1728, 1994.
3) Moore PA, Hersh EV : Local anesthetics:

Pharmacology and toxicity. Dent Clin N Am 54 : 587-599, 2010.

4) Becker DE, Reed KL : Local anesthetics: Review of pharmacological considerations. Anesth Prog 59 : 90-101, 2012.

5) Ikeda Y, Oda Y, Nakamura T, Takahashi R, Miyake W, Hase I, Asada A: Pharmacokinetics of lidocaine, bupivacaine, and levobupivacaine in plasma and brain in awake rats. Anesthesiology 112 : 1396-1403, 2010.

6) Kim EJ, Kim HY, Ahn JH : Neurotoxicity of local anesthetics in dentistry. J Dent Anesth Pain Med 20 : 55-61, 2020.

7) Badr N, Aps J : Narrative review: the evidence for neurotoxicity of dental local anesthetics. J Dent Anesth Pain Med 20 : 63-72, 2020.

8) Sorensen RG, Mahler HR : Calcium-stimulated adenosine triphosphatases in synaptic membranes. J Neurochem 37 : 1407-1418, 1981.

9) Nagy A, Shuster TA, Rosenberg MD : Adenosine triphosphatase activity at the external surface of chicken brain synaptosomes. J Neurochem 40 : 226-234, 1983.

10) Carafoli E, Brini M : Calcium pumps: Structural basis for and mechanism of calcium transmembrane transport. Curr Opin Chem Biol 4 : 152-161, 2000.

11) Dhalla NS, Zhao D : CELL MEMBRANE Ca^{2+}/Mg^{2+} ATPase. Prog Biophys Mol Biol 52 : 1-37, 1988.

12) Lin SC, Way EL : Characterization of calcium-activated and magnesium-activated ATPases of brain nerve endings. J Neurochem 42 : 1697-1706, 1984.

13) Garcia-Martin E, Gutiérrez-Merino C : Local anesthetics inhibit the Ca^{2+}, Mg^{2+}-ATPase activity of rat brain synaptosomes. J Neurochem 47 : 668-672, 1986.

14) Garcia-Martin E, Gutiérrez-Merino C : Modulation of the Ca^{2+}, Mg^{2+}-ATPase activity of synaptosomal plasma membrane by the local anesthetics dibucaine and lidocaine. J Neurochem 54 : 1238-1246, 1990.

15) Pottorf WJ, De Leon DD, Hessinger DA, Buchholz JN : Function of SERCA mediated calcium uptake and expression of SERCA3 in cerebral cortex from young and old rats. Brain Res 914 : 57-65, 2001.

16) Chifflet S, Torriglia A, Chiesa R, Tolosa S : A method for the determination of inorganic phosphate in the presence of labile organic phosphate and high concentrations of protein: Application to lens ATPases. Anal Biochem 168 : 1-4, 1988.

17) Sánchez GA, Di Croce DE, de la Cal C, Richard SB, Takara D : Differential mechanism of the effects of ester-type local anesthetics on sarcoplasmic reticulum Ca-ATPase. Naunyn-Schmiedeberg Arch Pharmacol 386 : 1061-1069, 2013.

18) Di Croce DE, Trinks PW, Grifo MB, Takara D, Sánchez GA : Drug action of benzocaine on the sarcoplasmic reticulum Ca-ATPase from fast-twitch skeletal muscle. Naunyn-Schmiedeberg Arch Pharmacol 388 : 1163-1170, 2015.

19) Di Croce DE, Trinks PW, de La Cal C, Sánchez GA, Takara D : Amide-type local anesthetics action on the sarcoplasmic reticulum Ca-ATPase from fast-twitch skeletal muscle. Naunyn-Schmiedeberg Arch Pharmacol 387 : 873-881, 2014.

20) Sánchez GA, Takara D, Alonso GL : Local anesthetics inhibit Ca-ATPase in masticatory muscles. J Dent Res 89 : 372-377, 2010.

21) Nozadze E, Arutinova N, Tsakadze L, Shioshvili L, Leladze M, Dzneladze S, Chkadua G : Molecular mechanism of Mg-ATPase activity. J Membr Biol 248 : 295-300, 2015.

22) Suzuki K, Kawada I, Iida A, Ono S, Deyama Y, Nishikata M, Matsumoto A : Inhibition of Na,K-ATPase activity by local anesthetics in rat brain and rabbit brain and kidney. In: Na/K-ATPase and related ATPases. Edited by Taniguchi K, Kaya S. Amsterdam, Elsevier Science BV. pp 747-750, 2000.