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EFECTO DEL AGONISTA BETA-3 ADRENÉRGICO EN MUSCULO ESQUELÉTICO

T E S I S

QUE PARA OBTENER EL GRADO DE

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P R E S E N T A

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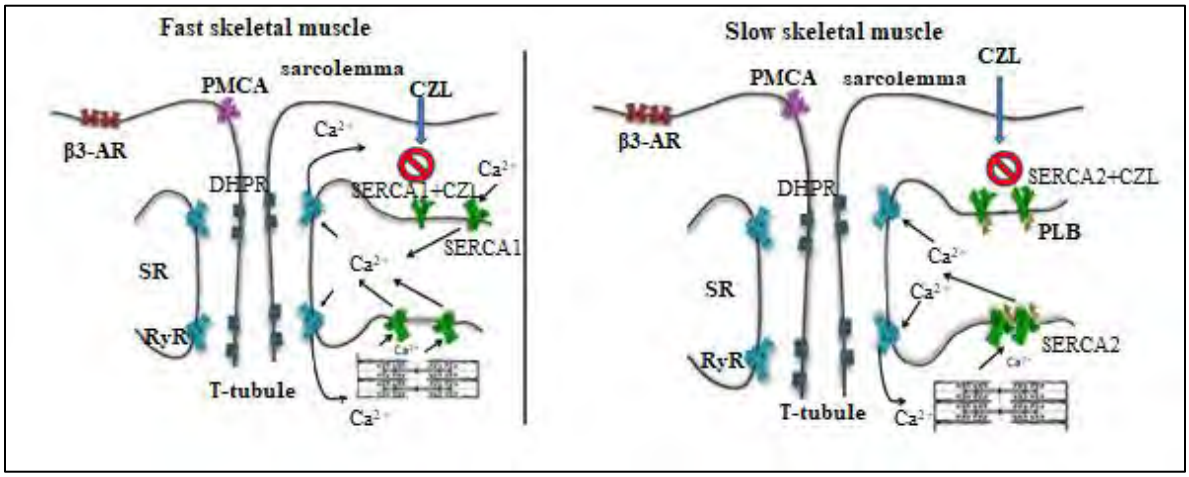
Contents

Abbreviations

- ADP = Adenosine Diphosphate
- AMP = Adenosine Monophosphate
- ARs = Adrenoceptors
- ATP = Adenosine Triphosphate
- CZL = Carazolol
- DHPR = Dihydropyridine receptor
- EDL = *Extensor Digitorum Longus*
- G-protein = Guanine nucleotide binding protein
- GDP = Guanosine Diphosphate
- GTP = Guanosine Triphosphate
- GPCR = G-protein coupled receptor
- P_i = inorganic phosphate
- PKA = Protein Kinase A
- PLB = Phospholamban
- PMCA = Plama membrane calcium ATPase
- SERCA = Sarco/endoplasmic reticulum calcium ATPase
- SLN = Sarcolipin
- SM = Skeletal Muscle
- SR = Sacrcoplasmic Reticulum
- T- tubule = Transverse Tubule
- Ti = Inactivation temperatura

Abstract

Carazolol (CZL) is a known agonist of β3 and antagonist of β1 and β2 adrenoceptors (AR), used in the animal production industry to improve meat quality by reducing animal stress and muscle proteolysis. Here we sought to better understand the direct effect CZL has on skeletal muscle. We study CZL effect on calcium (Ca^{2+}) regulation by enzymatic activity kinetics of the Ca^{2+}-ATPase (SERCA), in isolated sarcoplasmic reticulum (SR) from skeletal muscle (SM). Our studies show a 45% reduction in SERCA activity when SM is incubated with 0.03 mM CZL. Thermal analysis of SERCA activity with CZL shifted the transition temperature of inactivation (T_i) from T_i = 47 to 44 °C. When isolated SR from fast and slow SM is exposed to CZL, inhibition of SERCA occurs in a dose dependent manner. Slow and fast SM shift to a lower T_i in the presence of CZL and a second transition appears at < 40 °C. Our results suggests that CZL penetrates the plasma membrane and interacts with SERCA, thus having an important effect on skeletal muscle function by inhibiting SR Ca^{2+}-ATPase activity and, in consequence, a decrement in SR Ca^{2+}-release promoting further failure in muscle contraction.



Graphical abstract. Proposed intracellular interaction of CZL with SERCA1 and SERCA2. (SR), sarcoplasmic reticulum; (AR), adrenoceptor; (PMCA), plasma membrane Ca^{2+} ATPase; (DHPR), dihydropyridice receptor; (T-tubule), transverse tubule; (CZL), Carazolol; (RyR), ryanodine receptor; (PLB), phospholamban.

1. Introduction

1.1. Skeletal Muscle

In mammalian vertebrates, SM tissue is found abundantly and usually makes up around 40% of animals dry weight. This tissue has primarily evolved to perform contraction. By connecting to bones in the limbs and spine of animals and contracting, intricate coordinated activities can be done, giving rise to seemingly simple actions such as walking, maintaining body position, and holding or utilizing objects. In other words, through the action of skeletal muscle, vertebrate animals are able to interact and displace themselves in an environment directly. Unlike other muscle tissues such as cardiac muscle, skeletal muscle has no intrinsic ability for contraction, therefore stimulus for skeletal muscle activity is derived from voluntary nerve impulses.

1.2. Myofiber (types)

In mammals, skeletal muscle is composed of bundles of muscle cells known as myofibers, which are long and tubular. These thread like cells are connected at both ends to tendons which then connect to some part of a bone of the skeleton in an animal's body. The myofiber can be large (1-40 mm in length and 10-50 μM in width) and are multinucleated, arising from the fusion of myoblasts during embryonic development (Lodish *et al.,* 2000). These myofibers can be classified into certain types. Originally, distinction between myofiber types was based on color, arising from the concentration of myoglobin, where red myofibers possess a high concentration while white myofibers contain less (Needham, 1926). This distinction between fiber

types correlated with other physiological properties. White myofibers were confirmed to possess a fast contraction speed, fatigable and mainly glycolytic metabolism. On the contrary, red myofibers possess a slower contraction speed, resistance to fatigue and have mainly oxidative metabolism (Greising *et al.,* 2012). As a result, the white and red myofiber classification based on color advanced to one based on metabolic properties and contraction speed. Further research showed that the dichotomy classification of myofiber was inadequate since it was proven that myofibers can exist as a continuum between fast glycolytic and slow oxidative myofibers (Gollnick and Hodgson, 1986). However, in general myofibers can be classified as either fast or slow type.

Myofiber types are usually intermixed in skeletal muscle, however, some muscles can contain a predominant myofiber type. For example, in rat skeletal muscle, the *Extensor digitorum longus* (EDL) is a muscle located in the rat hindlimb that contains approximately 85% fast glycolytic type myofibers. Similarly, the *Soleus* muscle contains approximately 90% slow oxidative myofibers (Eng *et al.,* 2008). For this reason, these are known generally as fast and slow skeletal muscles, respectively. It is important to note that these proportions can be variable. Changes in myofiber type are seen during postnatal development. In rat *Soleus* muscle, one week old rat pups contain about 50% of the fast-glycolytic myofiber type. Within 1-2 months there is a drastic change in composition, shifting to about 10% of the fast-glycolytic type (Wigston and English, 1991). In addition, exercise training in skeletal muscle causes a shift from slow to fast myofiber types. Specifically, high intensity and high velocity training in skeletal muscle induces this in mammals (Wilson *et al.,* 2012). The exact causes of

these changes are unknown but the empirical data surrounding training and myofiber changes is strong.

1.3. Myofiber anatomy

The cytoplasm of mature myofibers is occupied mainly by the contractile machinery of the cell. It is made up of protein fibrils longitudinally arranged along the myofiber length into repeating structures known as sarcomeres, seen as the contractile unit of the muscle cell (Figure 1.1). The main components of the contractile machinery in myofibers are the thick and thin filaments, myosin and actin respectively. Contraction in myofibers and ultimately skeletal muscle, is brought about through the action of actin and myosin filaments sliding past one another. Myosin head groups contain ATPase hydrolytic activity. In addition, the myosin head group is able to bind to actin, forming cross-bridges between the two filaments. Current models suggest that the binding of ATP to myosin causes dissociation from actin filaments. The hydrolysis of ATP then causes a conformational change that displaces the myosin head to a further position on the actin filament. Hydrolyzed ATP by-products are released, ADP and $P_{i,}$ returning the myosin head to its original orientation. Successive ATP hydrolysis slides the actin filaments across the thick filament (Cooper, 2000). The interaction of myosin and actin in muscle is depressed by the regulatory proteins tropomyosin and troponin, associated to the actin filaments.

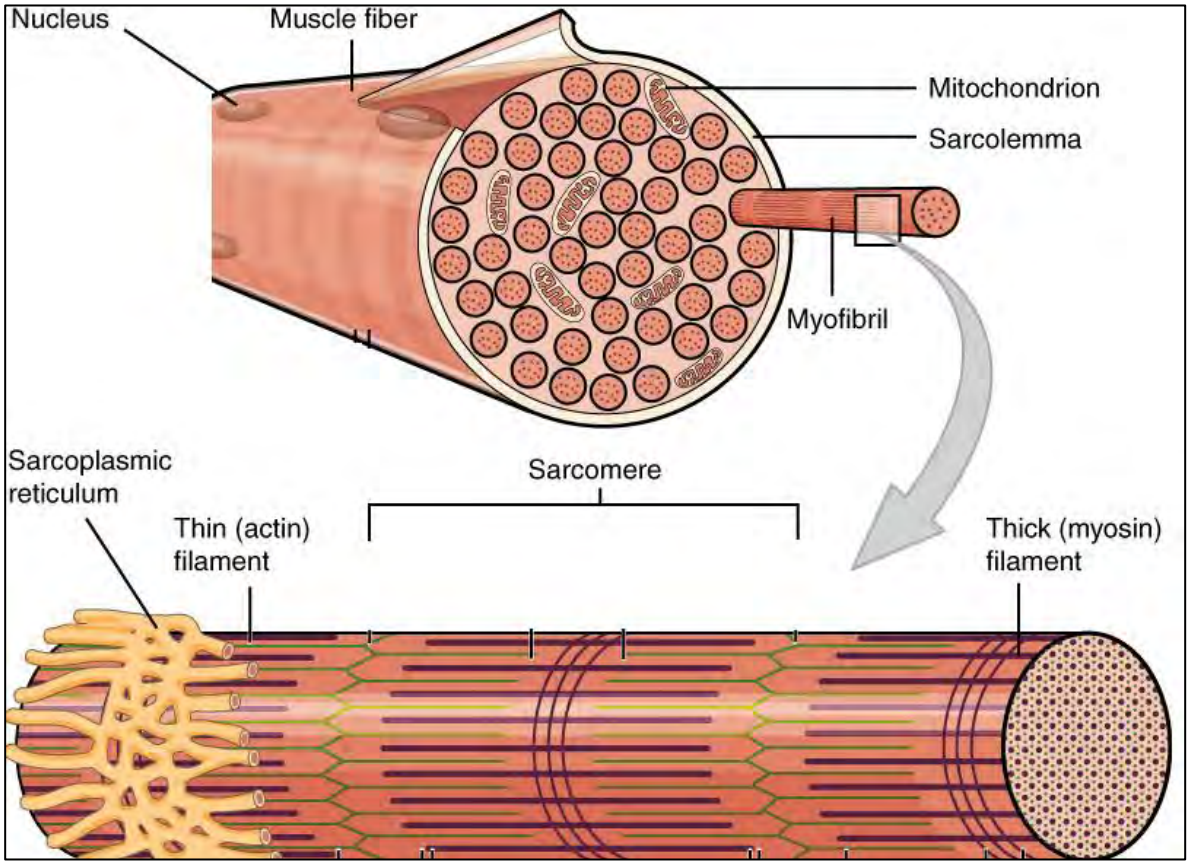


Figure 1.1. Muscle fiber anatomy. The majority of the internal space of the muscle fiber is occupied by protein fibrils which are mainly composed of the thick (myosin) and thin (actin) filaments. In addition, the position of the sarcoplasmic within the muscle fiber can be observed around the protein fibrils.

1.4. Tubular systems

Aside from the aforementioned contractile fibrils, the myofiber contains a network of interweaving membranes (Figure 1.2). Not until the use of electron microscopic studies were the details surrounding this structure resolved into two parts: the transverse tubules (T-tubules) and the sarcoplasmic reticulum (SR). T-tubules are regular cell membrane invaginations which are inserted perpendicular to the longitudinal axis of the myofiber. As a result, the membrane of the T-tubule is

continuous with the plasmalemma. The tubular system of invaginations is responsible for conducting the electrical signal required for contraction from the cell surface to internal areas of the myofiber, ensuring simultaneous contraction (Qusairia and Laporte, 2011). The SR is a specialized endoplasmic reticulum specific to muscle cells, but does not contain ribosomes (Ezerman and Ishikawa, 1967). It forms an internal membrane system of tubules and cisterns which are positioned transversely and longitudinally in the mammalian myofiber (Figure 1.2) (Schmalbruch, 1985). This specialized organelle functions as the site of Ca^{2+} storage, release and uptake during muscle contraction in SM. Calcium is an important second messenger necessary for the initiation of contraction in skeletal muscle. The details surrounding Ca^{2+} and contraction process are explained later on. Both the T-tubule and SR are specialized to ensure the simultaneous release of Ca^{2+} from the SR for contraction.

Since myofibers are relatively large structures, 50-100 μm in diameter and several millimeters to centimeters long, morphological adaptations are required to overcome the limitations size imposes on the signaling required for contraction (Qusairia and Laporte, 2011). In myofibers, the triad functions as a structure for efficient signal transduction in SM. The T-tubule forms a tight association with extensions of SR called terminal cisternae, this close association of the membranes form what is known as the triad. Electron microscopy studies reveal that even though these membranes are tightly associated in the triad, they remain physically separate (Figure 1.2).

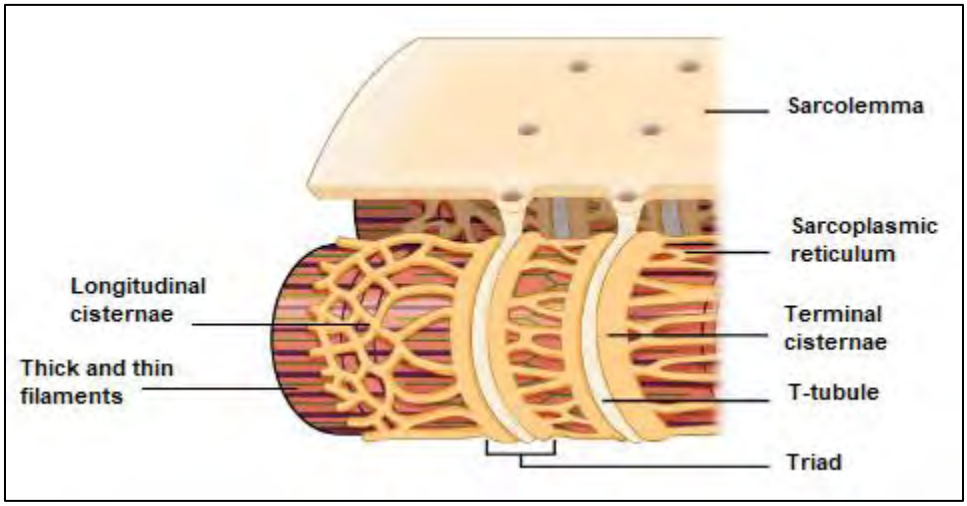


Figure 1.2. Structure and location of internal membranes in myofiber. T-tubule membrane extend from the sarcolemma and penetrate into the myofiber. Triad is formed by the association of SR on two sides of the T-tubule (Betts *et al.,* 2017).

1.5. Calcium in skeletal muscle contraction

Skeletal muscle contraction is initiated by nerve impulses, which stimulate Ca^{2+} release from SR. The signal for Ca^{2+} release begins at the endplate of the motor neuron axon as an action potential. This causes a liberation of acetylcholine from the motor axon terminal, which interact with acetylcholine receptors located on the myofiber plasmalemma initiating an action potential in the SM. The action potential spreads across the plasmalemma and into the interior of the myofiber along the T-tubules. As mentioned previously, the T-tubules are tightly associated with the SR. The arrival of the action potential induces Ca^{2+} release from the SR. The T-tubule voltage sensing dihydropyridine receptor (DHPR) induces Ca^{2+} release from the SR through protein-protein interaction with the Ca^{2+} release channel Ryanodine receptor (RyR). This causes an increase in intracellular Ca^{2+} from approximately 10^{-7} to 10^{-5} M

(Cooper, 2000). High Ca^{2+} concentration in myofiber initiates contraction by the action of the previously mentioned regulatory proteins tropomyosin and troponin (Figure 1.3). Tropomyosin is a filamentous protein that binds lengthwise along the actin filaments. In addition, tropomyosin is also associated with a complex of troponin proteins (Figure 1.3). Under resting condition, the low intracellular Ca^{2+} concentration causes the tropomyosin protein to block the site of interaction between actin and myosin. With SR Ca^{2+} release, the troponin complex shifts position of tropomyosin revealing the actin-myosin site of interaction allowing contraction to proceed (Figure 1.3), (Cooper, 2000). The contraction of the muscle generates the pulling force. The intracellular Ca^{2+} concentration is directly related to force production, therefore high Ca^{2+} levels in the myofiber results in an increase in force production. (Balnave and Allen, 1995). For this reason Ca^{2+} is considered the initiating factor for contraction.

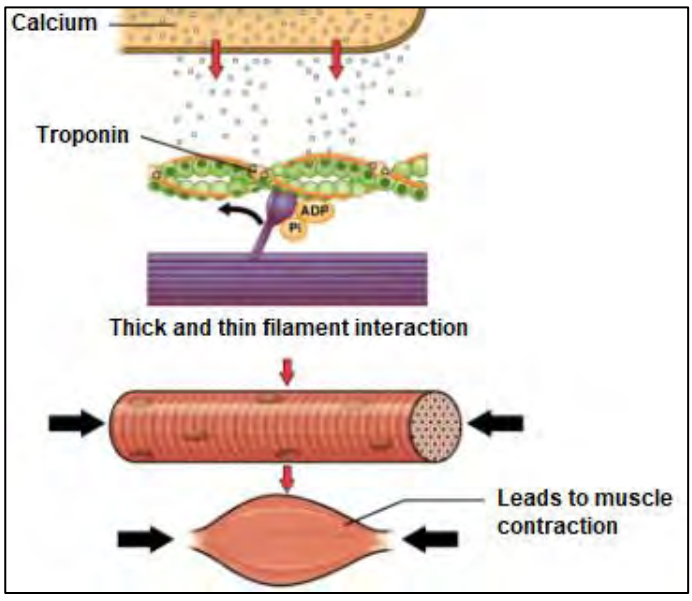


Figure 1.3. Contraction of a myofiber. Interaction of thick and thin filaments occurs as long as Ca2+ ions are released from the SR and as long as ATP is available. These steps lead to the contraction of the myofiber and overall leads to muscle contraction (Betts *et al.,* 2017).

1.6. Sarco (Endoplasmic) Reticulum Ca^{2+} -ATPase (SERCA)

When SR membrane is fragmented and isolated, small vesicles form from the resealing of the membrane. This crude preparation of SR can be separated into different fractions by gradient centrifugations. The light SR fraction has been shown to correspond to the longitudinal cisternae of the SR, portion of the SR that is not associated to the triad structure of the myofiber and extends lengthwise along the myofiber (Figure 1.2). These membranes have been studied biochemically and morphologically, revealing that the factor responsible for Ca^{2+} clearance is mainly present in the SR (Saito *et al.,* 1984). These studies demonstrate that the enzyme responsible for Ca^{2+} uptake in SM is SERCA (Figure 1.4). This enzyme is an integral membrane protein capable of transporting cytosolic calcium into the SR, against a concentration gradient using ATP as a substrate. In order for relaxation of SM to be achieved, Ca^{2+} must be cleared after massive release from SR stores following contraction (Figure 1.4). As a result, the SERCA pump is the factor directly responsible for SM relaxation Even though other calcium transport systems such as the plasma membrane calcium ATPase (PMCA) exist in skeletal muscle, they do not have a significant role in the process of calcium elimination from cytosol when compared to SERCA.

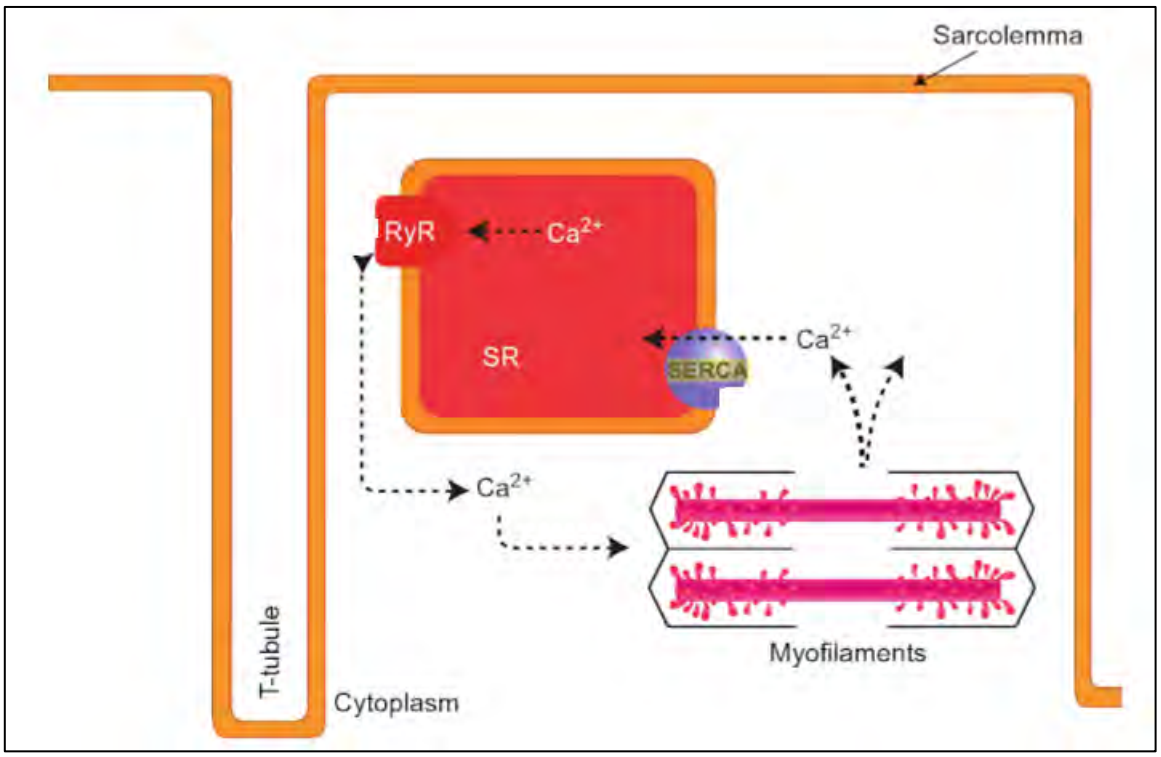


Figure 1.4. SERCA function during excitation-contraction coupling in skeletal muscle. Depolarization of the sarcolemma travels down the T-tubule, triggering the release of Ca^{2+} from the SR through the RyR (Ryanodine receptor) Ca^{2+} channel. The myofilaments initiate contraction once Ca^{2+} concentration is increased in the cytoplasm. In order for relaxation to occur, Ca^{2+} concentration must be reduced in the cytoplasm. This is mainly carried out by the ATP dependent uptake of calcium into the SR (sarcoplasmic reticulum) by SERCA (Adapted from Hill *et al.*, 2012).

SERCA has at least five isoforms present in mammalian tissues. The isoforms are a result of alternate splicing in at least three different genes. The SERCA1 isoform is found predominantly in fast skeletal muscle fibers. Alternate splicing generates two different isoforms, SERCA1a present in adult fibers and SERCA1b present as the primary calcium ATPase during development and in the neonatal, however this form is rapidly replaced after birth by SERCA1a (Brandl *et al.,* 1987). Similarly, the SERCA2 isoform can have its C-terminal alternatively spliced, producing two primary isoforms.

SERCA2a is expressed mainly in cardiac and slow skeletal muscle fibers, while the SERCA2b is found in non-muscle tissues and smooth muscle cells (Rossi and Dirksen, 2006). In all isoforms, SERCA is a monomer protein integrated in the SR membrane. Crystallographic studies demonstrate that the cytoplasmic region can generally be divided into three well defined domains: the actuator which is the site with major conformational changes during the reaction cycle of SERCA, phosphorylation and nucleotide binding domain, A, P and N respectively (Toyoshima *et al.,* 2000), (Figure 1.5). The calcium binding domain is located near the bilayer in the transmembrane portion of SERCA. Large conformational changes of the A, P and N sites occur as a response to calcium occupying the affinity site (Chen *et al.,* 2008).

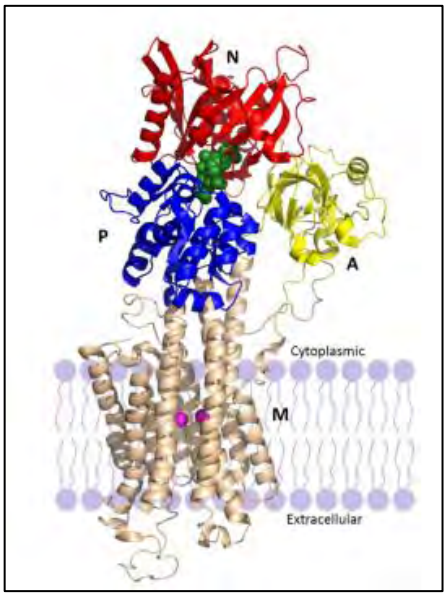


Figure 1.5. General structure of SERCA represented by cartoon model. A (actuator), N (nucleotide binding), P (phosphorylation) and M (transmembrane) domains are labeled. An ATP molecule is bound in the N domain, represented as green spheres. And calcium ions (pink) are bound to the cytoplasmic side of the membrane (Kotšubei, 2013).

1.7. SERCA regulation

The activity of SERCA is regulated mainly by proteolipids associated to the SR. A Proteolipid is a highly hydrophobic protein that contain a lipid moiety regularly and are soluble in organic solvents. It was first observed as a 31 amino acid peptide that isolates with SR preparation of rabbit skeletal muscle, because of this, it was named sarcolipin (SLN) (Wawrzynow *et al*., 1992). Even though it is highly abundant in SR, the function of SLN remained elusive until further studies showed that SERCA and SLN colocalize in the SR of adult skeletal muscle, and SLN associates directly with fast isoform SERCA1 (Figure 1.6). This suggested that SLN may function as a possible modulator of SERCA1. Later on, it was found that SLN can reduce the calcium affinity and transport of SERCA1 when both proteins are in a cell model expression system (Asahi *et al.,* 2002; Odermatt *et al.,* 1998). Similar to the expression of SERCA, SLN is highly expressed in fast skeletal muscle and to a lesser extent in slow skeletal and cardiac muscle, where instead another protein related to SLN, phospholamban (PLB), is expressed (Odermatt *et al.,* 1997). Additionally, *in vivo* studies evaluated the physiological significance of SLN in mice. SERCA activity rates of SLN null mice demonstrate an increase in activity and contractile kinetics, in skeletal muscles which normally express high levels of SLN (Tupling *et al.,* 2011). Proving that SLN in fact has a modulatory effect on SERCA1 activity in SM.

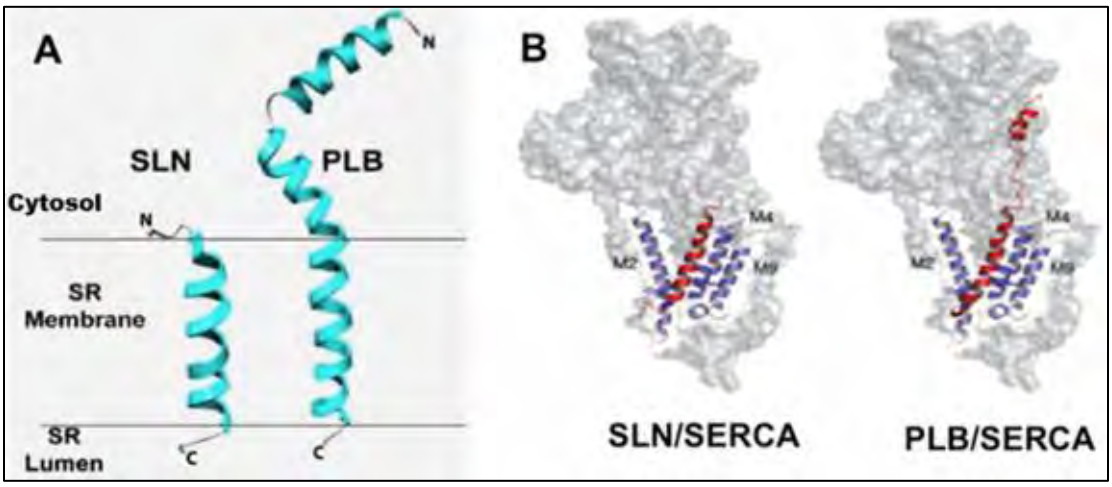


Figure 1.6. A) Comparison of SLN (sarcolipin) and PLB (phospholamban) structure in the SR (sarcoplasmic reticulum). B) Interaction of SLN and PLB with SERCA. Both SLN and PLB bind to the same transmembrane groove on SERCA. (Shaikh *et al.,* 2015).

The other proteolipid is the 52 amino acid residue homologue of SLN, PLB (Tada *et al.,* 1989), (Figure 1.6). As mentioned previously, PLB expression is generally restricted to cardiac and slow skeletal muscle fibers, where the SERCA2 isoform is predominantly expressed (Asahi *et al.,* 2002). The physiological role of PLB has been extensively studied in cardiac muscle. Research regarding isolated heart preparations show the SERCA2 isoform is regulated by PLB. β-adrenergic stimulation of cardiac muscle leads to phosphorylation of phospholamban resulting increased calcium affinity of SERCA2 and enhanced cardiac relaxation rates (Kranias *et al.,* 1985; Karczewski *et al.,* 1987). In addition, catecholamines, compounds which stimulate the β-adrenergic pathway, increase myocardial contractility as well. It was previously thought that this effect was a result of increased cardiac energy production, but this was quickly ruled out, since it could not explain the sudden increase in contractile augmentation. Once it was understood that cardiac relaxation is due to calcium uptake

by the SR, research was focused on possible factors located in the SR which could be altered by β-adrenergic stimulation. Eventually, researchers learned that phosphorylation of PLB by the cyclic AMP (Adenosine Monophosphate) dependent protein kinase A (PKA) or the calcium-calmodulin dependent protein kinase causes shifts in the calcium transport activity of SERCA (Katz, 1998). The mechanism of PLB SERCA2 inhibition is now understood to be a result of a direct interaction of the two proteins when PLB is un-phosphorylated. As a result, PLB is an endogenous regulator of the cardiac SERCA isoform, removed from the ATPase by phosphorylation, increasing activity (James *et al.,* 1989).

In the case of slow skeletal muscle fibers of most vertebrates, phospholamban is expressed at least in approximately equimolar amounts to the SERCA2 isoform (Ferrington, 2002). Presumably, PLB present in skeletal muscle functions similarly as in cardiac muscle. Ablation of PLB in a transgenic mouse model leads to significant changes in contractility of murine soleus muscle (Slack *et al.,* 1997). These experiments demonstrate that in PLB deficient soleus muscle has a 25% decrease in relaxation time as compared to wild type. The changes in relaxation time were not associated to an alteration of SERCA protein expression levels. The addition of isoproterenol, a β-adrenergic stimulator, to the PLB-deficient mice muscle showed that the relaxation time was unchanged, while wild type (WT) mice had a significant decrease in this parameter. This result suggests that PLB functions similarly in SM as in cardiac muscle and that PLB has a functional role in slow SM (Slack *et al.,* 1997).

1.8. Adrenoceptors

The adrenoceptors (AR) are a class of membrane spanning receptors which play a functional role in mediating the actions of the neurohormones norepinephrine and epinephrine released from sympathetic nerve endings and the adrenal medulla, respectively. Both these catecholamines regulate diverse physiological systems including: circulatory, metabolic, respiratory, and central nervous system, and are widespread throughout the body of mammals (Perez 2006).

The ARs are one of the first family of receptors to be extensively studied. An important development in the understanding of the physiological function of ARs comes from studies using isolated tissues and AR agonists. The 20th century American scientist Raymond Ahlquist determined that certain catecholamines can cause contraction or relaxation of uterus, pupils and cardiac muscle. In addition, he demonstrated that certain catecholamines can cause contraction or relaxation of smooth muscle depending on the dose and site of action. For example, norepinephrine caused smooth muscle contraction and epinephrine could cause both contraction and relaxation of smooth muscle (Perez 2006). With these observations, Ahlquist determined that these varying effects in tissues are a result of two distinct receptor subtypes, classified as α and β adrenergic receptors. In this case, the separation of the subtypes was determined by the catecholamine potency of the receptor, that is to say, in the rank order of the agonist potencies. Both the α and β ARs belong to a large super family of membrane receptors known as G-protein coupled receptors (GPCRs), which transmit signals to the interior of cells by associating to guanine nucleotide regulatory proteins known as G-proteins. Typical GPCRs such as the ARs, signal

through the binding of a first messenger, usually a hormone, to the extracellular side of the receptor. This in turn causes conformational changes in the receptor, that promotes the association between the receptor and the heterotrimeric G proteins conformed of the Gα and the Gβγ subunits (Figure 1.7). The coupling of the receptor and G proteins causes activation by catalyzing the exchange of GTP for GDP in the Gα subunit and dissociation from the heterotrimeric G-protein. Once separated, the free Gα and Gβγ subunits can regulate the activity of enzymatic effectors, such as adenylate cyclases, phosolipase C isoforms, and ion channels. These enzymatic effectors generate second messengers which control the activity of protein kinases involved in other metabolic pathways (Luttrell 2008), (Figure 1.8). Isolation and purification of ARs demonstrated that there are several distinct types of both α and β ARs. The primary structure is highly conserved between the subtypes, however they behave differently in pharmacological affinity for agonists and antagonists, as well as in the coupling pathways followed after hormone receptor interaction (Small *et al.,* 2003).

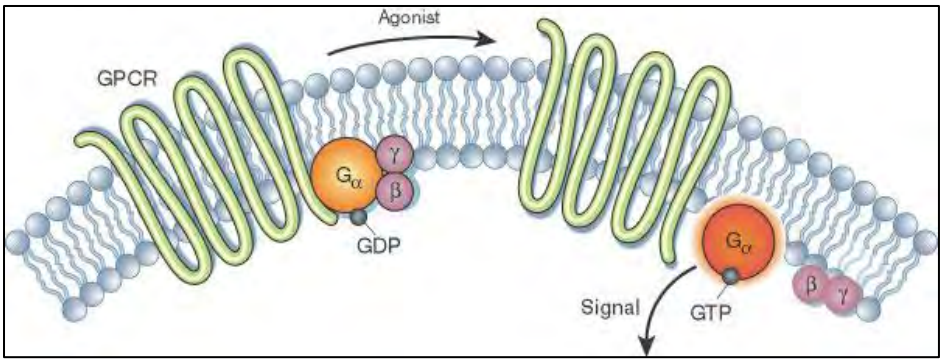


Figure 1.7. Basic GPCR (G-protein coupled receptor) model. The signaling model is made up of four principle components, the agonist, receptor, heterotrimeric G proteins (G_α, β, γ) and the effector enzyme. (Li *et al.,* 2002).

The predominant subtypes of AR in SM are the β-ARs, specifically the β2 subtype (Kim *et al.,* 1991). There are three β subtypes to date, the β1, β2 and β3 ARs (Lynch and Ryall, 2008). Even though there is a sparse population of α-ARs in SM, usually in higher proportions in highly vascularized SM, the main focus of AR research in SM is focused on the β subtype. This is mainly due to the anabolic effects observed when β-AR agonists were administered in animals. The use of β-AR agonists in animal production shows an increase in the quantity of muscle mass and decrease in the quantity of fat in the animal body. For this reason, these compounds are commonly referred to as repartitioning agents due to the change from fat deposition toward muscle formation. As mentioned previously, these actions are mediated through the membrane spanning β-ARs found in both adipose and skeletal muscle tissue. In the case of adipose tissue, the administration of β-AR agonists reduces lipid accumulation in adipocytes by increasing lipolysis and reducing lipogenesis (Muir, 1988). While in SM, the administration of β-AR agonists increases

the mass of skeletal muscle by causing an increase in protein synthesis and decrease in protein degradation, resulting in an overall shift towards deposition of protein in this tissue. As a result, there is an increase in the size of the tissue. Increase in tissue mass can be caused by two ways, either hyperplasia (increase in number of cells) or hypertrophy (enlargement of cells).

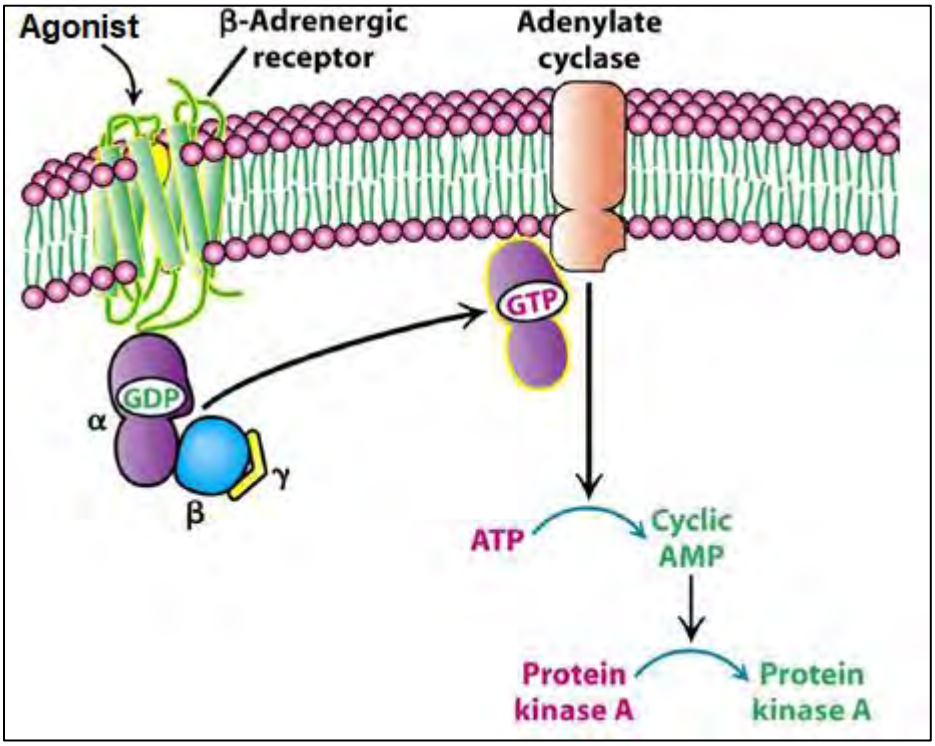


Figure 1.8. Characteristic β-AR signaling cascade. When stimulated by an appropriate agonist, β-AR activates adenylyl cyclase through the heterotrimeric G proteins producing cyclic AMP from ATP. The production of cyclic AMP activates protein kinase A, a well-established downstream agent of the β-ARs (Berg *et al.*, 2002).

The existence of β3-AR in SM has remained controversial, however a functional population involved in proteolysis inhibition has been suggested in other works through pharmacological studies (Sillence *et al*., 1993; Navegantes *et al.*, 2005). Furthermore, direct evidence of β3-AR expression in human SM has been confirmed by the use of specific monoclonal antibodies (Chamberlain et al., 1999). Still the specific role of β3-AR in SM remains elusive, though recent studies in human and animal models reveal that β3-AR play a role in the regulation of protein metabolism in SM (Puzzo *et al.,* 2016).

1.9. Carazolol

The drug CZL is a well-known potent agonist of the human and murine β3-AR (Méjean et al., 1995). For this, it has been used in human and veterinary medicine as an antihypertensive drug (Gregory and Wilkins, 1982; Keul et al., 1985). Even though most research involving CZL has been used systemically in animals, the effects in peripheral tissues such as SM remains to be explored. Previous research has demonstrated that β3 agonists such as CZL exerts an inhibitory action on proteolysis in rat slow SM but not in fast SM (Navegantes *et al.,* 2005). However the exact details surrounding β3 signaling in SM remain to be investigated. With more information, it is possible to reveal possible novel therapeutic targets that manipulate pathways beneficial to SM health.

In order to increase the yield, in this case SM mass, while also increasing feed efficiency, other β-AR agonists are used in the animal production industry such as clenbuterol and zilpaterol (Johnson *et al*., 2014), (Figure 1.9). These agonists target

other β-AR subtypes, however a similar hypertrophic effects occur in the SM of animals such as bovines when they are administered.

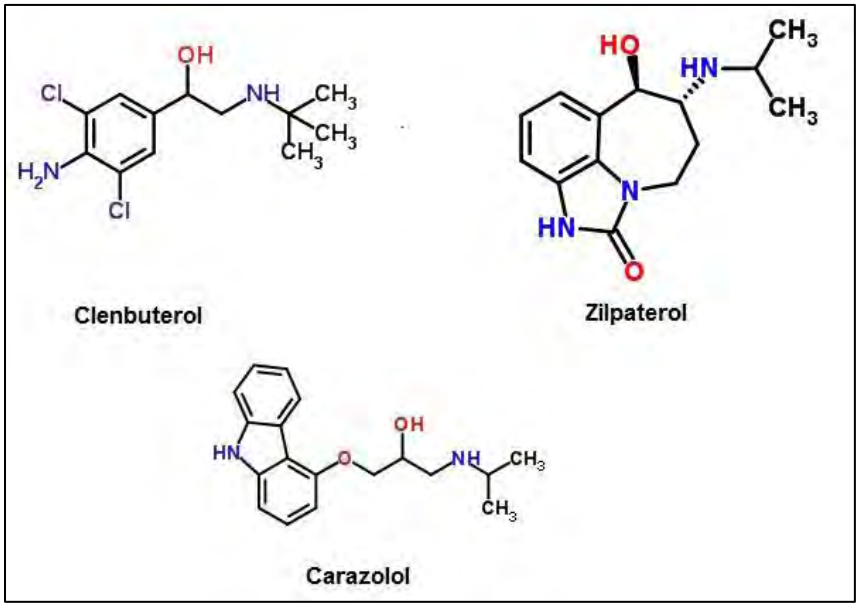


Figure 1.9. Structure of commonly used beta agonists including Carazolol

1.10. Temperature Studies on Enzyme Activity

For any reaction, a critical variable that determine the rate of reaction is temperature. In general, an increase in 10°C from room temperature doubles the rate of reaction for organic species in solution. In the late nineteenth century, the Swedish chemist Svante Arrhenius, was the first to formalize the relationship between temperature and the rate of reaction.

$$V = A_0 e^{-Ea/RT}$$

Where (V) is the rate of reaction, (A_0) is the pre-exponential factor, E_a is the activation energy, (R) is the gas constant and (T) is the absolute temperature. The pre-exponential factor in the Arrhenius equation is a factor that varies between 0 and 1, that gives the proportion of the potential reaction rate that can occur, given the kinetic state of the reactants. The product of RT is the average kinetic energy of the reactants, whereas temperature increases, the energy of the reactants increases as well (DeLong *et al*., 2017). This equation was originally applied to explain the temperature dependence of chemical reaction rates in controlled settings, but it has also been applied to biological rates such as enzyme-catalyzed reactions.

The inactivation of enzymes can be brought about by different methods such as temperature, pH, ionic strength, and chemical inactivation. Among these, the most practical way to achieve inactivation is by thermal inactivation. This type of inactivation occurs at high temperatures. Since enzymes are stable at the ambient temperature for the organism from which they are obtained, they lose activity when temperature is increased to higher levels (Klibanov, 1983).

At the molecular level, the process of thermal inactivation can be understood as an unfolding of the molecule (Tanford, 1968). Under normal conditions, the enzyme has a native structure with a catalytically active site, maintained by a variety of noncovalent forces such as: hydrogen bonds, hydrophobic, ionic and van der Waals interactions (Klibanov, 1983). As temperature increases, these forces weaken and the protein molecule unfolds, resulting in a less ordered conformation (Figure 1.10).

The region where a substrate molecule binds and undergoes a chemical reaction in an enzyme is known as the active site of an enzyme. The active site is

made up of amino acid residues that form temporary bonds with the substrate, catalyzing the reaction. These amino acid residues are brought together in the native three-dimensional structure of the enzyme. The unfolding that occurs during a steady increase in temperature disassembles the active site and as a result leads to enzyme inactivation (Klibanov, 1983).

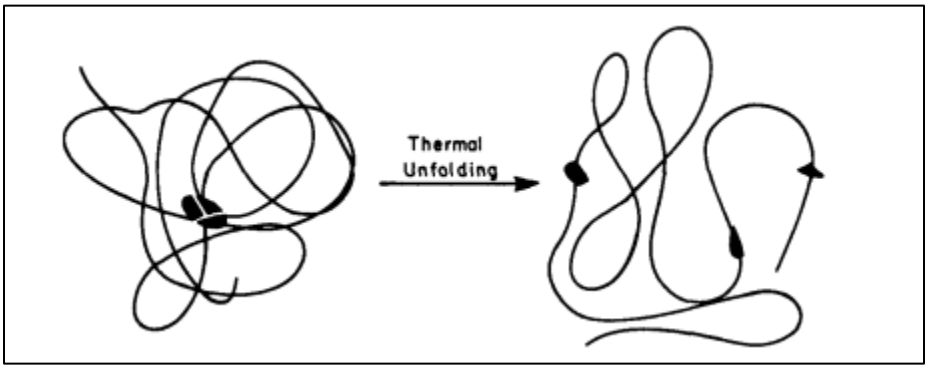


Figure 1.10. Representation of thermal unfolding an enzyme. Filled region represents the active site of the enzyme. Prior to thermal unfolding, enzyme presents a defined three-dimensional structure. After thermal unfolding, structure is lost, as well as functionality of active site (Klibanov, 1983)

2. Problem statement

CZL is a known β1 and β2 antagonist administered in the animal production industry to improve meat quality by reducing stress. Previous research has demonstrated that there is a hypertrophic effect in SM when utilized in whole animals. In addition, when CZL is incubated in isolated skeletal muscle, evidence suggests that there is a negative effect in the force production and fatigue resistance (also referred to as physiological activity) (Rodriquez Correa, 2013). Although these effects are clear, the intracellular action of CZL in SM remains to be evaluated.

2.1. Objective

The objective of the present work is to determine the intracellular effect of the drug CZL on the activity of SERCA in fast and slow skeletal muscle.

2.2. Hypothesis

Previous studies have demonstrated that there is a loss in physiological activity when CZL is incubated directly in both fast and slow isolated skeletal muscle. Physiological activity in SM is directly related with the calcium release and uptake that occurs during contraction. It is proposed that this change is a result of an alteration of calcium regulation in the SR as a result of SERCA enzyme activity inhibition with CZL, since the SERCA pump is the protein directly responsible for calcium uptake after contraction.

3. Materials and Methods

3.1. Animals.

All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources of the United States and approved by the Ethics Committee of the School of Medicine of the National Autonomous University of Mexico (UNAM) (NOM-062-ZOO1999).

3.2. Isolation of Sarcoplasmic Reticulum.

Male Wistar rats weighing 280-300 g (6 ± 1 week) were euthanized by cervical dislocation on the same day of SR isolation. The EDL and Soleus were quickly isolated. Membranes were obtained from fast and slow skeletal muscle (EDL and soleus muscles respectively). The isolation was performed by differential centrifugation in a discontinuous sucrose gradient as previously described (Becker *et al.,* 2004; Ortega *et al.,* 2000). The microsomal fraction was placed in a sucrose gradient of 25%, 27.5% 32% and 35% (w/v) and centrifuged at 23,000 rpm for 16 h. The interface 27.5/32% was identified as Light SR, since it contains the maximum ATPase activity stimulated by Ca^{2+} (SERCA). The protein concentration of each sample was determined using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, USA) with BSA as the standard.

3.3. SERCA1 hydrolytic activity.

Total ATPase activity was measured by the colorimetric determination of P_i using malachite green as described previously (Lanzetta et al., 1979). Aliquots of light SR 5 μg protein/ml were incubated in a solution containing; 100 mM KCl, 5 mM $MgCl_2$, 5 mM NaN_3, 20 mM Tris-malate, 0.1 mM $CaCl_2$ and 0.33 mM ATP (pH 7). The reaction was stopped after 30 minutes with a solution containing 0.045% malachite green hydrochloride/ 4.2% ammonium molybdate in 4 N HCl, 0.25 ml sodium citrate (34%) and 0.8 ml Triton X-100 for each 100 ml of solution, and the absorbance was read at 660 nm. These experiments were performed in the absence and presence of CZL using the concentrations indicated in the figure legend.

3.4. Thermal inactivation of ATPase activity.

LSR membranes (0.05 mg/ml) were incubated in a solution containing: 100 mM KCl, 5 mM $MgCl_2$, 5 mM NaN_3, 20 mM Tris-malate, 0.1 mM $CaCl_2$ and 0.33 mM ATP (pH 7) in a thermal cycler at a heating rate of 1°C/min as described previously (Lepock *et al.,* 1990; Vázquez *et al.,* 2016). Samples were recovered at time intervals of 1 min from 25 to 70°C, as indicated in the corresponding figure legends, and placed on ice for approximately the same amount of time until all samples were collected. The ATPase activity at room temperature (25°C) was measured, and an inactivation curve was generated. The first derivative of the inactivation curve was used to determine the T_i (inactivation temperature) which is defined as the temperature recorded for half inactivation. For all inactivation experiments, the curves are not symmetrical, and therefore, the T_i does not correspond exactly to half of the curve.

3.5. Immunoblotting

Microsomal fractions of EDL and Soleus muscles were isolated from male Wistar rats weighting 280-300g. The isolated microsomal fractions were analysed by dot blot assay. Approximately 5μl of the same amount of protein concentration of each preparation were placed on a nitrocellulose membrane and let dry for 15 min. The membrane was blocked with 5% non-fat dry milk (Bio-Rad) and incubated with β3-AR (M-20; Polyclonal, Santa Cruz) with a dilution of 1:500 for one hour at room temperature. Thereafter washing and incubation with the corresponding peroxidase labelled antibody was carried out for 30 minutes. Signal for dot-blot were developed using Millipore Immobilon system.

3.6. Statistical analysis.

All values are expressed as means standard deviation of the mean (S.D.M.). The experiments with n=2, refer to the absolute error of mean (A.E.M.).

4. Results

4.1. Effect of incubation of intact isolated muscle with Carazolol (CZL) on SERCA1.

Isolated EDL muscle was placed in the experimental chamber incubated in Krebs solution, an isotonic physiological solution containing: 0.135 M NaCl, 0.005 M KCL, 0.001 M MgCl, 0.015 M $NaHCO_3$, 0.001 M Na_2HPO_4, 0.011 M $C_6H_{12}O_6$ with 0.03 mM CZL for 10 min. After incubation, muscle was washed to remove CZL and were used to perform SR membrane isolation. Figure 2.1 shows the effect of CZL incubation

on SERCA1 hydrolytic activity. Figure 2.1A displays the normalized thermal inactivation of SERCA; interestingly, the activity was 45% lower for the muscle previously incubated with CZL at 25 °C. To better visualize the Ti in these cases, the first derivative of the thermal inactivation curves was generated (Figure 2.1B), for control muscle the Ti was 47 °C ± 0.7 (n=5) and 44ºC for muscle pre-incubated with CZL.

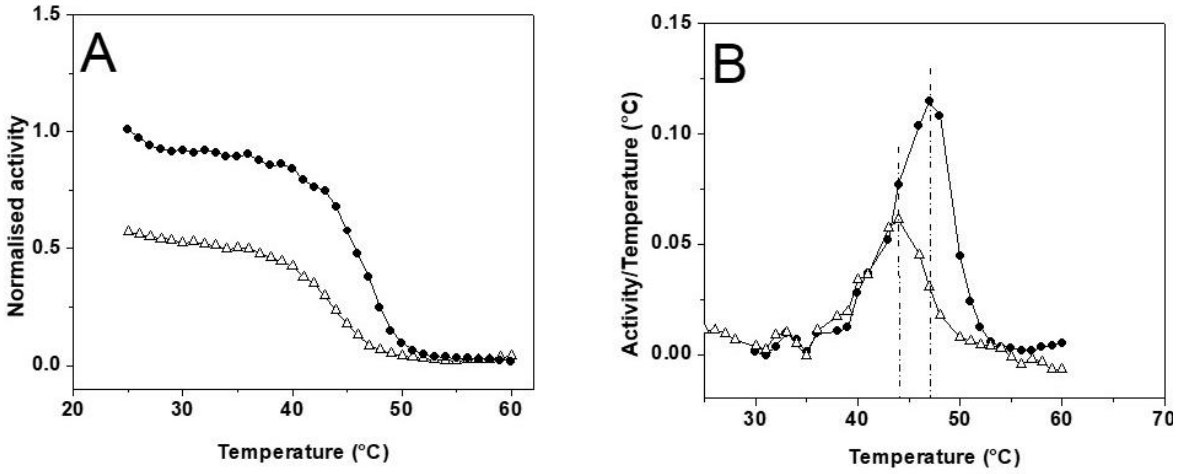


Figure 2.1. SERCA1 activity and SERCA1 activity previously incubated with 0.03 mM Carazolol. A) Thermal inactivation curve of SERCA1 hydrolytic activity where (●) control; (n =4), (△) (n =1) corresponds to SERCA1 activity isolated form EDL muscle previously incubated with 0.03mM Carazolol prior to SR isolation. B) Derivative curve of thermal inactivation of SERCA1 ATP hydrolytic activity as a function of increased temperature. Control (●), (△) corresponds to SERCA1 activity isolated form EDL muscle previously incubated with 0.03mM Carazolol prior to SR isolation.

4.2. Effect of Carazolol concentration on SERCA1 and SERCA2 isolated from EDL and Soleus muscle

CZL has a dose-dependent effect on SERCA1 and SERCA2 isolated from EDL and soleus muscle. Figure 2.2A reveals the dose dependent effect of CZL on SERCA1 activity from EDL muscle. The curve in figure 2.2A shows that approximately while 50% ± 19 (n=3) of the activity is lost with 0.5 mM CZL with respect to control. Figure 2.2B reveals the dose dependent effect of CZL on SERCA2 activity from soleus muscle as well. With 0.5 mM CZL, 67% ± 17 (n=4) of activity is lost with respect to control.

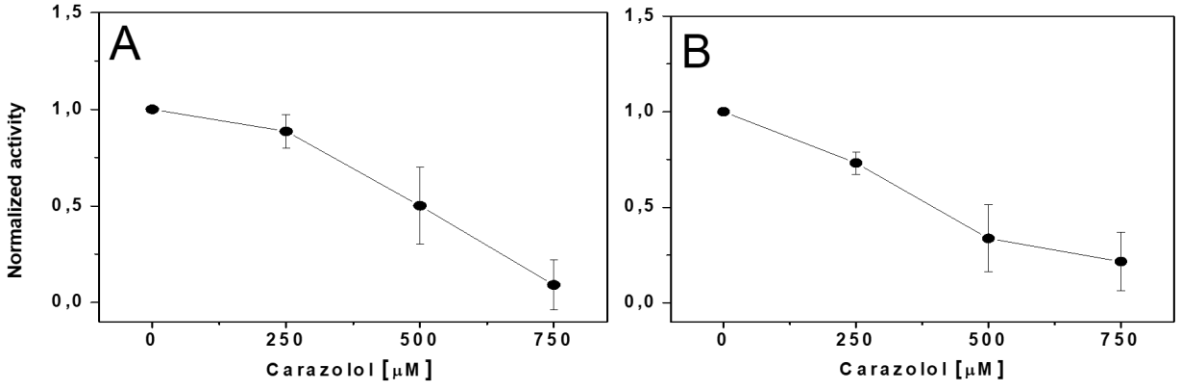


Figure 2.2. Effect of Carazolol on SERCA1 and SERCA2. A) Carazolol concentration effect on SERCA1 hydrolytic activity (n=3). B) Carazolol concentration effect on SERCA2 hydrolytic activity (n=4). Reaction was incubated for 30 minutes for each concentration indicated. The values of the activity were normalized to the reaction without CZL.

4.3. Enzyme kinetics of SERCA1 and SERCA2 in the presence of CZL

The kinetics of the SERCA1 isoform where first performed under normal conditions to observe the behaviour of the enzyme under control conditions. Subsequently, the kinetics of SERCA1 with CZL were carried out. Figure 2.3A demonstrates the kinetics of SERCA1 in the presence of 0.25 and 0.5mM, where activities were inhibited by 24% ± 4 and 37% ± 8 (n=3) respectively. Similarly, figure 2.3B displays the kinetics of SERCA2 in the absence and presence of 0.25 and 0.5mM CZL, at 30 min the activity is inhibited by 41% ± 7 and 76% ± 9 (n=4) respectively.

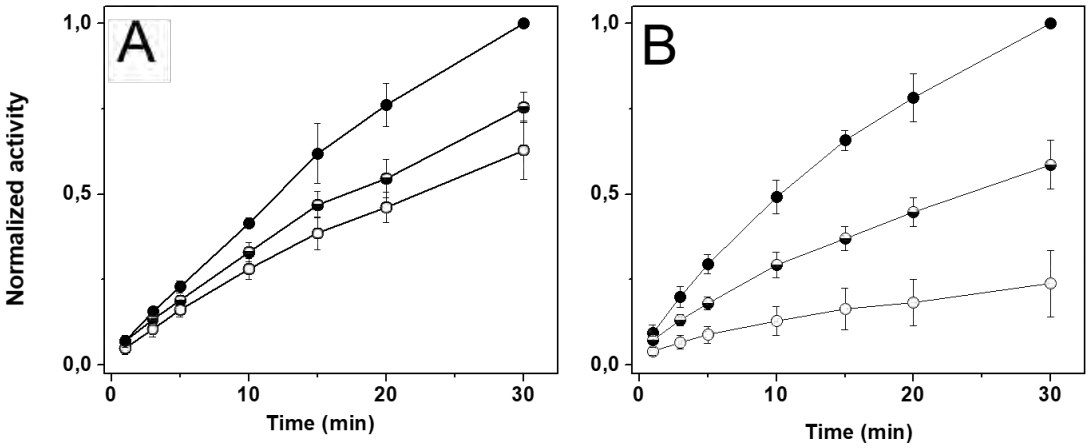


Figure 2.3. A) Enzyme kinetics of SERCA1 (●) control; n =3, (◒) 0.25mM; n=3 and (○) 0.5mM Carazolol; n=3. B) Enzyme kinetics of SERCA2 (●) Control; n =4, (◒) 0.25mM; n=4 and (○) 0.5mM Carazolol; n =4. The values for the activity were normalized to those of maximal activity at 30 min reaction of the control.

4.4. Thermal inactivation analysis of SERCA1

To observe if CZL inhibition was due to a direct interaction with SERCA1 a thermal inactivation analysis was performed. The thermal inactivation curves in Figure 2.4A displays the inactivation of SERCA in the absence and presence of 0.25 and 0.5 mM CZL. Figure 2.4B represents the first derivative of the thermal inactivation curves of hydrolytic activity from control SERCA1 with a T_i at 47°C ± 0.7 (n=5). In the presence of CZL two well-defined transitions were observed; in 0.25mM CZL the Ti was at 45ºC ± 1.5 and at 32ºC ± 0.1 (n=3) ; in 0.5mM CZL the Ti was at 43ºC ± 0.7 and at 37ºC ± 0.3 (A.E.M., n=2).

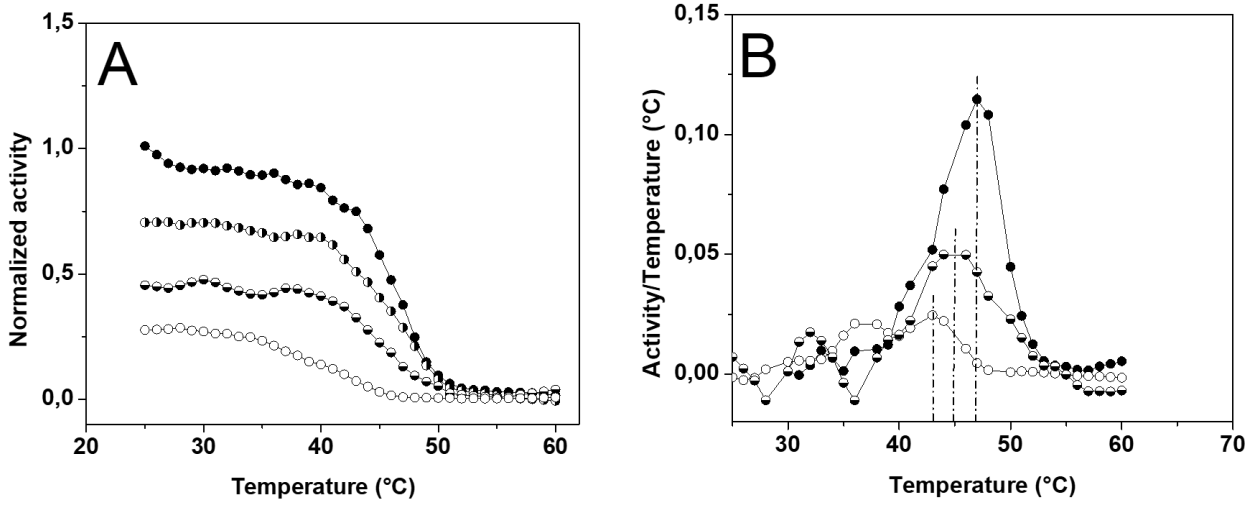


Figure 2.4. A) Represents thermal inactivation curves of SERCA1 hydrolytic activity (●) Control; n=4, (◗) 0.1mM; n =2, (◒) 0.25 mM; n =3 and (○) 0.5 mM CZL; n=2. B) Derivative curve of thermal inactivation of SERCA1 ATP hydrolytic activity as a function of increased temperature. Control (●), 0.25 mM (◒) and (○) 0.5 mM Carazolol. The values for the activity were normalized to those of maximal activity at 30 min reaction of the control.

4.5. Thermal inactivation analysis of SERCA2

Once again, to observe if CZL inhibition was due to a direct interaction with SERCA2 a thermal inactivation analysis was performed. The thermal inactivation curves in Figure 2.5A displays the inactivation of SERCA2 in the absence and in the presence of 0.25 and 0.5 mM CZL. Figure 2.5B represents the first derivative of the thermal inactivation curves of hydrolytic activity from SERCA2 with a T_i at 43°C ± 1.6 (n=5). In the presence of CZL two well-defined transitions were observed; with 0.25 mM CZL the Ti was at 44ºC ± 1.3 and 37ºC ± 1.8; (n=4); with 0.5 mM CZL the Ti was at 41ºC ± 0.7 and at 36ºC ± 0.3 (A.E.M., n=2).

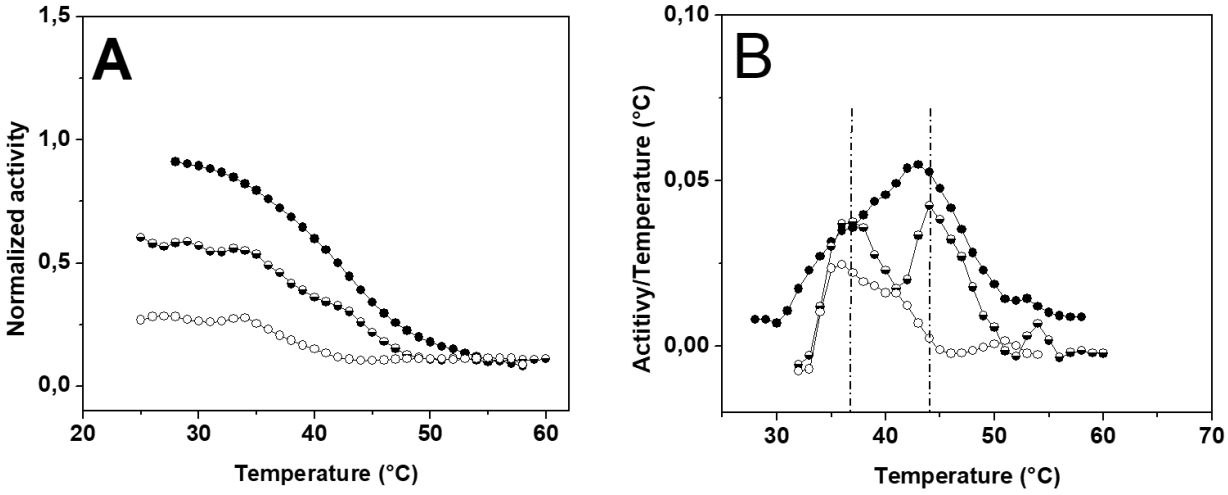


Figure 2.5. A) Represents thermal inactivation curves of SERCA2 hydrolytic activity (●) Control; n =5, (◒) 0.25 mM; (n=4) and (○) 0.5 mM Carazolol; (n=2). B) Derivative curve of thermal inactivation of SERCA2 ATP hydrolytic activity as a function of increased temperature. Control (●), 0.25 mM (◒) and (○) 0.5 mM Carazolol. The values for the activity were normalized to those of maximal activity at 30 min reaction of the control.

4.6. β3 adrenoceptors in skeletal muscle

The presence of β3-AR in SM is confirmed through the use of a dot blot assay as described in methods. Figure 2.6 shows both types of SM contain similar concentrations of β3-AR. We used isolated white adipose tissue as a positive control and BSA as a negative control for β3-AR. Fast and slow SM contains 20% less signal than white adipose tissue, known to have a significant amount β3-AR.

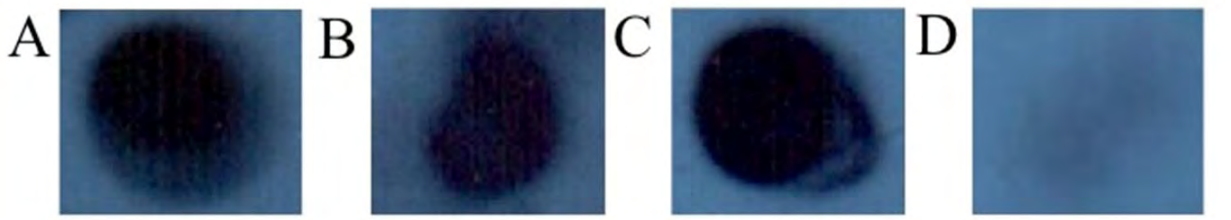


Figure 2.6. Dot blot analysis of β3 adrenoceptors. Dots shown are representative of different tissues with same concentrations (1mg/1ml) A) Corresponds to Soleus muscle, B) EDL muscle, C) white adipose tissue D) BSA. White adipose tissue serves as a positive control for β3 and BSA as a negative control.

5. Discussion

The known action of CZL as a drug which reduces the negative side effects of stress on skeletal muscle, has been observed to cause a decrease in muscle tension and proteolytic activity, bringing about an overall increase in skeletal muscle mass (Warris and Lister, 1982). It is assumed that these effects are mediated exclusively through the antagonistic action on β1 and β2-AR. However, from our results, 1) SR isolated from intact skeletal muscle exposed to CZL results in inhibition of SERCA hydrolytic activity, 2) there is an inhibitory effect of CZL on both SERCA1 and SERCA2 isoform from isolated SR, 3) SERCA1 and SERCA2 differ in their thermal denaturation profile which corresponds to differences in structure, 4) direct interaction of CZL on both SERCA isoforms as determined by thermal analysis, 5) β3-AR are present in both fast and slow SM. This data leads us to consider that CZL is able to cross to the inner side of the SM fibre and interact with SERCA in the SR of muscle. With this in mind, there exists a possible physiological explanation involving SERCA which explains the loss in force production and fatigue observed in our study of muscle.

5.1. Carazolol effect on SERCA in intact SM

The application of CZL to the external solution of SM would imply that the inhibition of SERCA we observe is mediated exclusively through the myoplasm β3-AR. However, when thermal stability of SERCA hydrolytic activity is evaluated when CZL is applied externally to intact SM and washed, the T_i of SERCA is clearly shifted to a lower temperature as compared to control, indicating that the nature of the

interaction between SERCA and CZL is direct. This result signifies that CZL is able to penetrate the Sarcolemma (cell membrane of muscle fiber). Other studies have demonstrated that this is not an isolated case. Dihydropyridine receptor antagonists are surface level drugs that have been shown to penetrate the plasma membrane and interact with SERCA (Ortega *et al.,* 1997). In rat SM Ca^{2+} uptake variation in fast and slow twitch fibers arise from differences in SERCA1 and SERCA2. Therefore, it is likely to observe dissimilarities when incubated with CZL, especially since the two SERCA isoforms present differences in structure, functionality and regulation. However in both cases it is assumed that inhibition is a result of a non-competitive allosteric inhibition. This is evidenced by the change in in T_i observed when SERCA is incubated with CZL. Even though we do not have direct evidence for this claim, but it can be inferred form the molecular structure of CZL that inhibition would be non-competitive and allosteric seeing as though it does not possess or even resemble a complementary structure to the SERCA active site.

5.2. Carazolol effect on SERCA activity

Previous studies have proven that SERCA isoforms present qualitatively similar enzymatic properties (Lytton *et al.,* 1992). The differences seen in Ca^{2+} transport arise from their expression pattern and in the specific regulation present in the different SM types. It is well established that SERCA2 isoform is regulated by the small molecular weight protein phospholamban (PLB) located in the SR of cardiac and slow muscle (Movesian *et al.,* 1992). The activity of SERCA2 depends on the phosphorylation state

of PLB. Dephosphorylated PLB binds and inhibits Ca^{2+} transport, in its phosphorylated state PLB relieves inhibition, increasing the relaxation rate of contraction (Colyer and Wang, 1991). In this study, both control SERCA isoforms present a similar activity. However, in the study of SR incubated directly with CZL, it can be seen that SERCA inhibition behaves differently depending on the isoform. When incubated with the same concentration of CZL, SERCA2 is inhibited approximately two times more when activity is normalized. A possible interpretation of this data is that CZL is inhibiting the two isoforms via different mechanisms, since activity is reduced differently.

5.3. Direct effect of CZL on SERCA1 and SERCA2

The thermal analysis curves generated with the incubation of CZL reveals some interesting information about SERCA activity. A negative shift of $\triangle T_i$=2°C occurs when CZL 0.25 mM is incubated with SERCA1, and is further reduced with increasing CZL concentration. This indicates that thermal stability is lowered in a concentration dependent manner which relates with protein conformational changes in SERCA1 with CZL. A comparison of the SERCA 1 and SERCA2 from Slow SM thermal inactivation profiles has not been evaluated until now. Control SERCA2 presents a T_i= 43°C ± 1.6, further proving that the two isoforms are structurally different as they vary in their thermal inactivation profiles. In both isoforms, it can be said that thermal stability decreases. Since activity is directly related to structure, the shifts in T_i signify that denaturalization is achieved at lower temperatures when incubated with CZL. Therefore, the addition of CZL is somehow affecting the ability to resist thermal denaturalization in both SERCA isoforms. Interestingly, the addition of CZL causes the

activity under control conditions to separate into two thermodynamically distinct activities. A possible explanation for this is that CZL interaction with SERCA2 results in the formation of two populations, one as a monomeric unit and the other as a more stable and active oligomer. There are evidences that SERCA 2 requires aggregation through PLB in the SR membrane in order to become fully active (Lennon *et al.,* 1999). This suggests that CZL interferes with the association of SERCA2, most likely through a SERCA2 conformational change obstructing the association with PLB. This new data would then correlate with SERCA2 existing as an oligomer, whose aggregation state is regulated under physiological conditions.

5.4. *β3 adrenoceptors (β3-AR) in skeletal muscle*

It is well established that β3-AR are present in white and brown adipocytes (Matteis *et al.,* 2002). Ligand binding studies have shown that membranes from adipose tissue and *soleus* muscle have identical binding sites for a known β3-AR ligand, suggesting that there is a population of β3-ARs in muscle. (Sillence *et al*., 1993). Immunoassays confirm that β3-AR are located in SM in general as well (Chamberlain *et al.,* 1999). In this study, we confirm the presence of the β3-AR and demonstrate that both fast and slow skeletal muscle have similar concentrations. Additionally, chronic pharmacological *in vivo* experiments with mice suggest that muscle force increases with β3 agonists, assumed to be as a result of β3-AR signalling in SM (Puzzo *et al.,* 2016). However, CZL, a compound known to exert its action through β3-AR has an inhibitory effect on SM mechanical properties when added directly to the isolated rat SM. The contradictory result observed may be due to a

different target of CZL when applied systemically (*in vivo)*, since a direct exposure of SM to CZL has strong inhibitory effect on muscle force and decrease fatigue resistance for both slow and fast SM. It is yet to be evaluated whether said detrimental effect in both SM types is truly explained through the β3-AR pathway given that CZL is a β3 agonist. But as we have seen with our results, CZL has an action independent of the β3-AR pathway through its direct interaction with SERCA.

6. Conclusion

In conclusion, we have seen that CZL a β3-AR agonist used in health and animal production has a deleterious effect on SM function that can be explained by a direct interaction with SERCA, an intracellular membrane protein in charge of muscle relaxation.

The finding that CZL has a strong effect on slow SM fatigue provides us with a pharmacological tool to investigate the molecular nature of the Slow SM resistance to fatigue. Furthermore, since our findings reveals that CZL is inhibiting SERCA2 and there is evidence of fatigue in soleus as well, these results provide strong evidence for theories which explain that the physiological cause of fatigue arises from the modification of SERCA function. Additional research is required to explore this idea, but CZL could be potentially used to explore this idea.

The fact that a CZL a β-AR agonist has a deleterious action in SM brings into question the use of such drugs in the animal production industry. As mentioned previously, these drugs are currently used and administered in commonly consumed

meat products. If these drugs are able to be transmitted to people through consumption of contaminated meat products, these harmful effects in SM could potentially be presenting themselves in people who consume these products.

Spanish Summary of Thesis

Resumen

El Carazolol (CZL) es un agonista de e los adrenoceptores (AR) β3 del y antagonista de los AR β1 y β2, que se ha utilizado en la industria de producción animal para mejorar la calidad de la carne a través de la reducción del estrés de los animales durante su transportación además de que se le conoce un efecto inhibitorio de la proteólisis del músculo. En el presente trabajo estudiamos el efecto directo del CZL en músculo esquelético a través de s efecto sobre la actividad enzimática de la ATPasa de calcio del retículo sarcoplásmico del músculo (SERCA). El Retículo sarcoplásmico (RS) aislado del músculo esquelético previamente incubado con 0.03mM CZL, pero ausente durante la determinación de la actividad de SERCA, resultó en una reducción de la actividad de SERCA del 45%. Los resultados del análisis térmico de la actividad de SERCA muestra que la temperatura de inactivación (Ti) varía de 47 ºC para la SERCA control a 44 ºC para la SERCA en presencia de CZL. Cuando el RS retículo sarcoplásmico tanto del músculo rápido como del lento son expuestos a CZL, la inhibición de SERCA ocurre de manera dosis dependiente. La Ti de SERCA tanto en el músculo esquelético rápido como en el lento disminuye en presencia de CZL y aparece una segunda transición a temperaturas < 40°C. Nuestros resultados indican que el CZL penetra la membrana plasmática e interactúa con SERCA, teniendo así un efecto importante sobre la función del músculo esquelético a través de la inhibición de la SERCA y en consecuencia, hay un potencial decremento del calcio liberado del SR necesario para que el músculo realice la contracción.

Introducción

En los vertebrados, el tejido muscular es el más abundante. La función principal del músculo esquelético ha evolucionado para realizar principalmente la contracción voluntaria.

El músculo esquelético se compone de conjuntos de células alargas de forma tubular conocidas como fibras. Las fibras se pueden clasificar en ciertos tipos dependiendo de sus propiedades fisiológicas asociadas con su velocidad de contracción. Existen dos tipos principales de fibras; el tipo rápido con metabolismo glucolítico y el tipo lento con metabolismo oxidativo, entre ellas existe una gran variedad de fibras intermedias, dependiendo de la especie. El citoplasma de las fibras musculares está ocupado principalmente por las proteínas de la maquinaria contráctil rodeada po la membrana plasmática o Sarcolema (SL) que se invagina de manera periódica para alcanzar la parte más interna de la fibra muscular, esas invaginaciones se conocen como los Túbulos transversos (TT) y son las responsable de conducir la señal para iniciar la contracción desde la superficie hasta el interior de la célula. Adicionalmente, el interior de la fibra contiene una red de membranas especializadas que funcionan como un sitio de almacenaje de calcio, para su liberación y captación en el proceso de contracción/relajación.

La SERCA es una enzima de membrana que hidroliza ATP para transportar calcio del citoplasma al interior del RS en contra de un gradiente de concentración.

Existen varias isoformas de SERCA que se expresan en diferentes tejidos y en diferentes etapas de la vida. La isoforma SERCA1 se expresa en las fibras de músculo esquelético rápido, mientras que la isoforma SERCA2 se expresa en el corazón y en las fibras de músculo esquelético lento. Las actividades de SERCA1 y SERCA2 son regulados por sarcolipina y fosfolambán respectivamente.

Los adrenoceptores son una clase de receptores de membrana plasmática que se activan con las hormonas norepinefrina y epinefrina. Existen dos tipos principales de adrenoceptores, tipo α y β. En el músculo esquelético existe en forma mayoritaria los subtipos $\beta 1$ $\beta 2$ y $\beta 3$. El Carazolol es un fármaco conocido como agonista adrenérgico del tipo $\beta 3$. Es utilizado en medicina veterinaria como un fármaco antihipertensivo.

La inactivación térmica de las enzimas es una manera de estudiar cambios estructurales de las mismas cuando están en presencia de ligandos que afectan su actividad. Al nivel molecular, la inactivación térmica de una enzima se puede entender como una pérdida de la estructura tridimensional de la estructura nativa de la proteína, terminando en un estado desplegado al azar. Como resultado, la actividad enzimática se pierde con el incremento en la temperatura, siguiendo el principio de Arrhenius.

2. Planteamiento del problema

El CZL es un β bloqueador que se administra en la producción animal para mejorar la calidad de la carne al reducir el estrés animal. Investigaciones previas sugieren que existe un efecto hipertrófico en el músculo esquelético cuando es

administrado en animales al reducir la proteólisis muscular. Sin embargo, cuando el CZL es incubado en músculo esquelético aislado, resulta en un efecto negativo en la actividad mecánica. Aunque estos efectos han sido documentados a nivel del organismo y del tejido, el mecanismo molecular involucrado en la acción intracelular de CZL en la fibra muscular queda por ser evaluado.

2.1. Objetivo

Determinar el efecto del Carazolol sobre la actividad de la SERCA en el Retículo Sarcoplásmico aislado de músculo esquelético rápido y lento.

2.2. Hipótesis

Estudios previos demuestran que hay una perdida en la actividad mecánica del músculo rápido y lento aislado cuando el Carazolol es incubado directamente en el músculo aislado. La actividad fisiológica del músculo esquelético está directamente relacionado con la liberación y captación de calcio que ocurre durante la contracción/relajación. En la presente tesis se propone que el efecto del CZL sobre las propiedades mecánica es el resultado de una alteración en la regulación de calcio en el retículo sarcoplásmico como un resultado de la inhibición de la actividad enzimática de SERCA, debido a que SERCA es la proteína directamente responsable por la captación de calcio después de la contracción.

3. Materiales y métodos

3.1. Animales.

Todos los procedimientos fueron realizados de acuerdo con la Guía para el Cuidado y Uso de Animales de Laboratorio del Instituto de Recursos

Animales para Laboratorio de los Estados Unidos y aprobado por el Comité de Ética de la Facultad de Medicina de la Universidad Nacional Autónoma de México (UNAM) (NOM-062-ZOO1999).

3.2. Aislamineto del Retículo Sarcoplásmico

Ratas macho de la sepa Wistar con peso entre 280-300 g fueron sacrificadas por dislocación cervical el mismo día que se aisló el retículo sarcoplásmico. El músculo *Extensor Digitorum Longus* y *Sóleo* fueron aislados rápidamente. Las membranas fueron obtenidas del músculo esquelético rápido y lento (EDL y Sóleo respectivamente) por separado. El aislamiento fue realizado por centrifugación diferencial en un gradiente discontinuo de sacarosa como ha sido descrito previamente (Becker *et al.,* 2004; Ortega *et al.,* 2000. La fracción microsomal fue puesta sobre un gradiente de sacarosa compuesto de 25%, 27.5% 32% y 35% (w/v) y centrifugado a 23,000 rpm por 16h. La fracción en la interface 27.5/32% fue identificado como retículo sarcoplásmico ligero (RSL), debido a que contiene una actividad máxima de ATPasa estimulado por calcio (SERCA). La concentración de proteína de cada muestra fue determinado con el reactivo de ensayo de proteína Coomassie Plus (Pierce, Rockford, IL, USA) con BSA como estándar.

3.3. Actividad hidrolítica de SERCA1.

La actividad total de ATPasa fue medida por la determinación colorimétrica de P_i utilizando el verde de malaquita como se describió previamente (Lanzetta et al., 1979). Alícuotas de 5μg protein/ml fueron incubados en una solución que contiene en mM: 100 KCl, 5 $MgCl_2$, 5 NaN_3, 20 Tris-malato, 0.1 $CaCl_2$ y 0.33 ATP (pH 7). La

reacción fue detenida con una solución que contiene 0.045% verde de malaquita clorhidrato/ 4.2% molibdato de amonio en 4N HCL, 0.25 ml en citrato de sodio (34%) y 0.8ml Triton X-100 por cada 100ml de solución, y la absorbancia fue leída a 660 nm. Estos experimentos fueron realizados en ausencia y presencia de CZL utilizando las concentraciones indicados en la leyenda de figura.

3.4. Inactivación térmica de la actividad de ATPasa.

Las membranas del RSL (0.05mg/ml) fueron incubados en una solución que contiene en mM: 100 KCl, 5 $MgCl_2$, 5 NaN_3, 20 Tris-malato 0.1 $CaCl_2$ y 0.33 ATP (pH 7) en un termociclador a una velocidad de calentamiento de 1°C/min como se describió previamente (Lepock *et al.,* 1990; Vázquez *et al.,* 2016). Las muestras fueron recuperadas a intervalos de 1 minuto desde 25 a 70°C, como es indicado en las leyendas de figuras correspondientes, y puesto sobre hielo por aproximadamente el mismo tiempo hasta que todas las muestras fueron recuperadas. La actividad de ATPasa fue determiada a temperatura ambiente (25°C). La primera derivada de la curva de inactivación fue utilizada para determinar la temperatura de inactivación. En todos los experimentos de inactivación, las curvas no son simétricas y en consecuencia, la temperatura de inactivación (Ti) no corresponde exactamente a la mitad del área bajo la curva.

3.5. Inmunoblot

Las fracciones microsomales de EDL y sóleo fueron aisladas de ratas macho Wistar con peso entre 280-300g. Las fracciones microsmales fueron analizados por dot-blot. Aproximadamente 5μl de cada preparación con aproximadamente la misma

concentración de proteína fueron puestas sobre una membrana de nitrocelulosa y dejaron secar por 15 minutos. La membrana se bloqueó con 5% de leche en polvo sin grasa (Bio-Rad) e incubado con el anticuerpo anti-β3-AR (M-20; Polyclonal, Santa Cruz) a una dilución de1:500 por una hora a temperatura ambiente. Después, es lavado e incubado con el anticuerpo correspondiente marcado con peroxidasa durante 30 minutos. La señal del dot-blot se obtuvo usando el sistema Millipore Immobilon.

3.6. Análisis estadístico

Todos los valores son expresados como la desviación estándar de la media. Para experimentos con n=2, se refiere al error absoluto de la media.

4. Resultados

4.1. Efecto de la incubación de músculo aislado intacto con Carazolol sobre SERCA1.

El EDL aislado fue puesto en una cámara experimenta incubado con solución Krebs y 0.03mM CZL por 10 minutos. Después de la incubación, el músculo fue lavado para quitar CZL. Estos músculos fueron usados para el aislamiento de retículo sarcoplásmico. La figura 2.1 muestra el efecto de la incubación con CZL sobre la actividad hidrolítica de SERCA1. La figura 2.1A señala la inactivación térmica normalizada de SERCA; la actividad se redujo 45% para los músculos previamente incubado con CZL. Para mejor visualizar la temperatura de inactivación en estos casos, la primera derivada de las curvas de inactivación térmicas se presenta en la (Figura 2.1B), para el músculo control la temperatura de inactivación fue 47°C ± 0.7 (n=5) y 44ºC para el músculo pre-incubado con CZL.

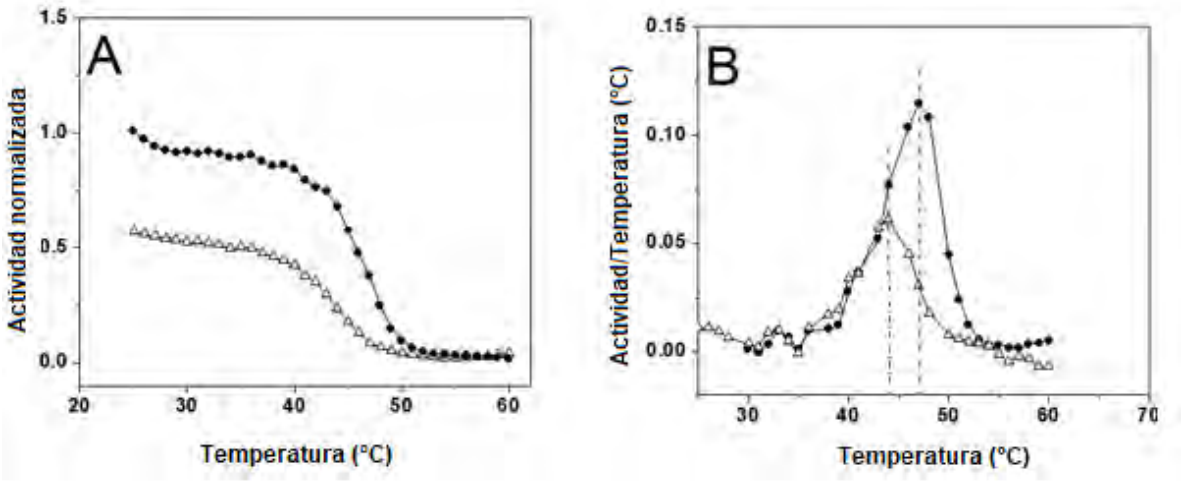


Figura 2.1 Actividad de SERCA1 previamente incubado con 0.03mM Carazolol. A) Curva de inactivación térmica de la actividad de SERCA1 donde (●) control; n =4, (△) corresponde a la actividad de SERCA1 aislado del EDL previamente incubado con 0.03mM Carazolol antes del aislamiento de retículo sarcoplásmico.

4.2. Efecto del Carazolol sobre SERCA1 y SERCA2 aislados de EDL y sóleo.

El Carazolol tiene un efecto dosis dependiente sobre SERCA1 y SERCA2 determinadas en RSL aislado del EDL y sóleo. La figura 2.2 revela el efecto dosis dependiente del Carazolol sobre la actividad de SERCA1 del músculo EDL. La curva en la figura 2.2A muestra que aproximadamente el 50% ± 19 (n=3) de la actividad se redujo con 0.5mM CZL con respecto a l a actividad de la SERCA control. La figura 2.2B revela el efecto dosis dependiente del Carazolol sobre la actividad de SERCA2 proveniente del músculo de sóleo. A la concentración de 0.5mM CZL la actividad se redujo 67% ± 17 (n=4) con respecto a la SERCA control.

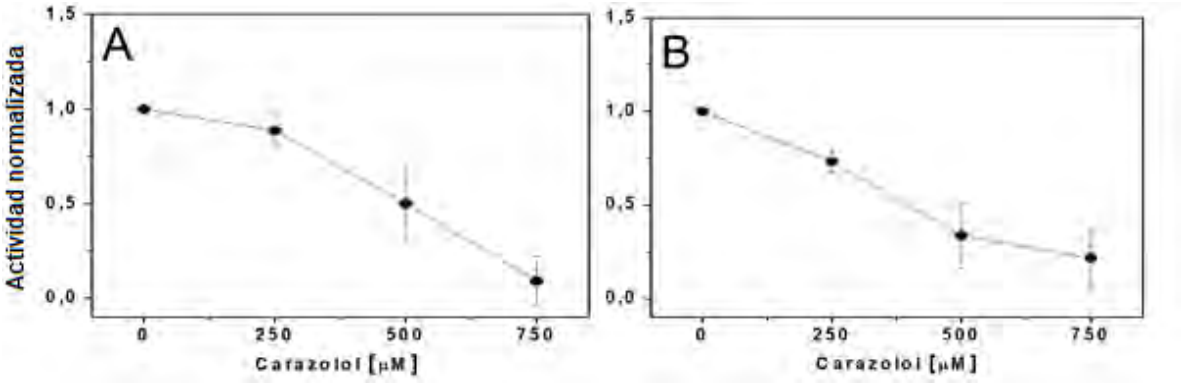


Figura 2.2 Efecto del Carazolol sobre SERCA1 Y SERCA2. A) Efecto de concentración del Carazolol sobre la actividad hidrolítica de SERCA1 (n=3). B) Efecto de concentración del Carazolol sobre la actividad hidrolítica de SERCA2 (n=4).

4.3. Cinética enzimática de SERCA1 y SERCA2 en la presencia de Carazolol.

La Figura 2.3 muestra la cinética de la SERCA en presencia de CZL. Figura 2.3A muestra la cinética de SERCA1 en presencia de 0.25 y 0.5mM, donde la actividad se inhibió en 24% ± 4 y 37% ± 8 (n = 3) respectivamente. De forma similar, la figura 2.3B muestra la cinética de SERCA2 en ausencia y presencia de CZL de 0.25 y 0.5mM, a los 30 minutos la actividad se inhibió 41% ± 7 y 76% ± 9 (n = 4) respectivamente.

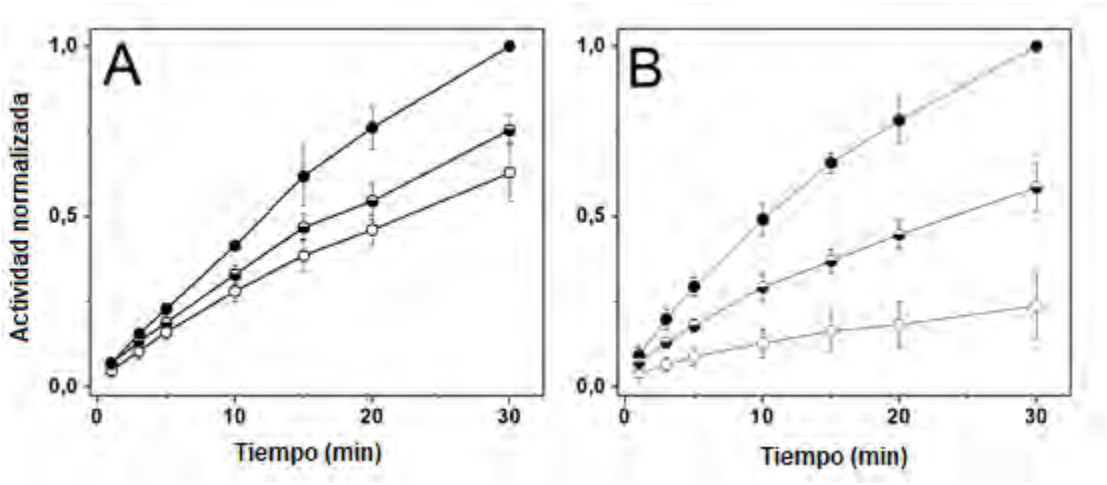


Figura 2.3. A) Cinética enzimática de SERCA1 (●) control; n =3, (◒) 0.25mM; n=3 y (○) 0.5mM Carazolol; n=3. B) Cinética enzimática de SERCA2 (•) Control; n =4, (◒) 0.25mM; n=4 and (○) 0.5mM Carazolol; n =4.

4.4. Análisis de la inactivación térmica de SERCA1

Para observar si la inhibición de CZL se debía a una interacción directa con SERCA1, se realizó un análisis de la inactivación térmica (Figura 2.4). Las curvas de inactivación térmica en la figura 2.4A muestran la inactivación de SERCA en ausencia y presencia de CZL de 0.25 y 0.5mM. La figura 2.4B representa la primera derivada de las curvas de inactivación térmica de la actividad hidrolítica del control SERCA1 con un T_i a 47°C ± 0.7 (n = 5). En presencia de CZL se observan dos transiciones; a 0.25mM CZL la temperatura de transición fue a 45ºC ± 1.5 y a 32ºC ± 0.1 (n = 3); a 0.5mM CZL la temperatura de transición fue a 43ºC ± 0.7 y a 37ºC ± 0.3 (A.E.M., n = 2).

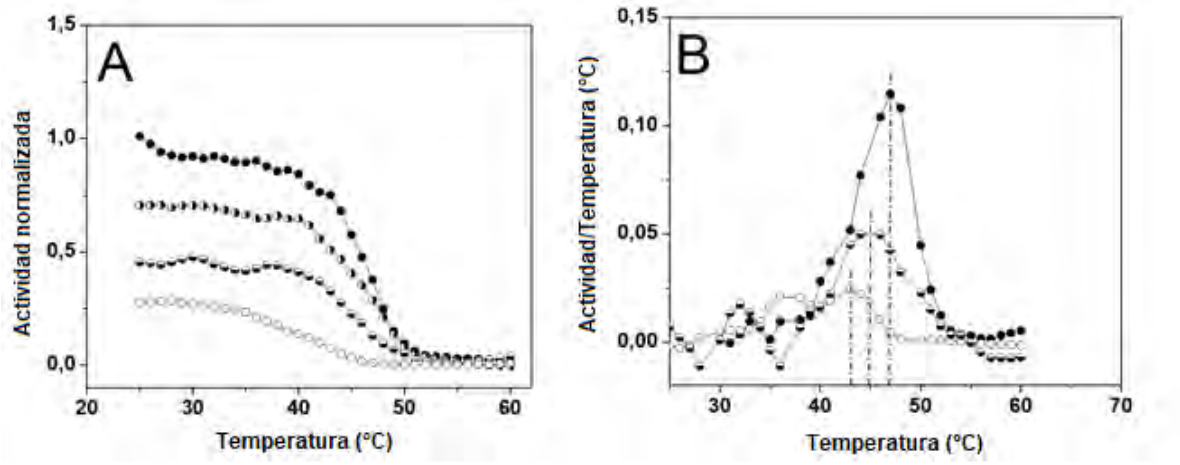


Figura 2.4. A) Representa las curvas de inactivación térmica de la actividad hidrolítica de SERCA1 (●) Control; n=4, 0.1mM; n=2, (◒) 0.25mM; n=3 y (○) CZL 0.5mM; n=2. B) Primera derivada de la inactivación térmica de la actividad hidrolítica de ATP de SERCA1 en función del aumento de la temperatura. Control (●), 0.25mM (◒) y (○) 0.5mM Carazolol.

4.5. Análisis de la inactivación térmica de SERCA2

Para observar si la inhibición de CZL se debía a una interacción directa con SERCA2, se realizó un análisis de la inactivación térmica (Figura 2.5). Las curvas de inactivación térmica en la figura 5A muestra la inactivación de SERCA2 en ausencia y presencia de CZL de 0.25 y 0.5mM. La Figura 2.5B representa la primera derivada de las curvas de inactivación térmica de la actividad hidrolítica de SERCA2 con una Ti a 43°C ± 1.6 (n = 5). En presencia de CZL se observaron dos transiciones bien definidas; a 0.25mM CZL la Ti fue a 44ºC ± 1.3 y 37ºC ± 1.8; n = 4; a 0.5mM CZL la Ti fue a 41ºC ± 0.7 y a 36ºC ± 0.3 (A.E.M., n = 2).

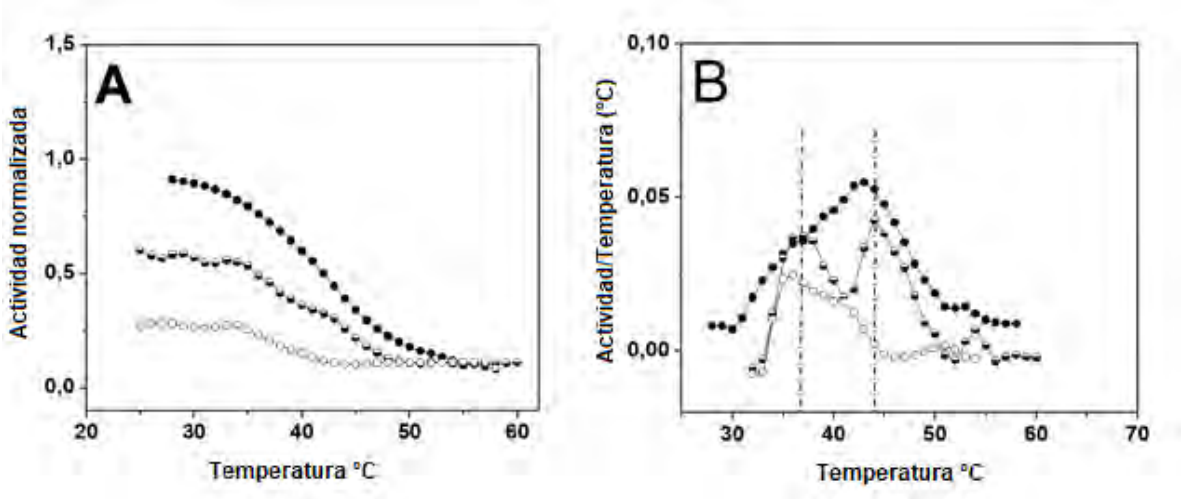


Figura 2.5. A) Representa las curvas de inactivación térmica de la actividad hidrolítica de SERCA2 (●), Control; n = 5, (◒) 0.25mM; n = 4 y (○) 0.5mM Carazolol; n = 2. B) Curva derivada de la inactivación térmica de la actividad hidrolítica de ATP de SERCA2 en función del aumento de la temperatura. Control (●), 0.25mM (◒) y (○) 0.5mM Carazolol.

4.6. Adrenoceptores β3 en músculo esquelético

La presencia de β3-AR en músculo esquelético se confirma mediante el uso de un ensayo de dot blot como se describe en la sección de materiales y materiales y métodos La Figura 6 muestra que ambos tipos de músculo esquelético contienen concentraciones similares de β3-AR. Utilizamos tejido adiposo como control positivo y BSA como control negativo para β3-AR. El músculo esquelético rápido y lento contiene un 20% menos de señal que el tejido adiposo, que se sabe que tiene una cantidad significativa de β3-AR.

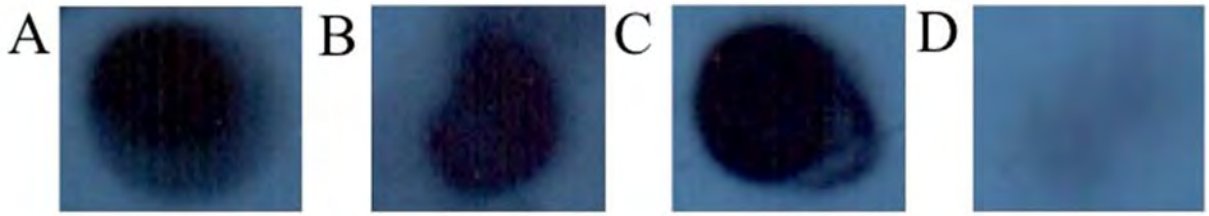


Figura 2.6. Dot blot análisis de los adrenoceptores β3. Se muestran blots representativas de diferentes tejidos con las mismas concentraciones (mg / ml) A) Corresponde al músculo soleo, B) al músculo EDL, C) al tejido adiposo blanco D) BSA. El tejido adiposo blanco sirve como control positivo para β3 y BSA como control negativo.

5. Discusión

Se ha observado que la acción conocida del Carazolol como fármaco que reduce los efectos secundarios negativos del estrés en el músculo esquelético provoca una disminución de la tensión muscular y de la actividad proteolítica, lo que provoca un aumento general de la masa muscular esquelética (Warris y Lister, 1982). . Se supone que estos efectos están mediados exclusivamente por la acción antagonista sobre β1 y β2-AR. Sin embargo, de nuestros resultados, 1) En el RS aislado del músculo esquelético y expuesto a CZL resulta en la inhibición de SERCA, 2) hay un efecto inhibidor de CZL en SERCA1 y SERCA2, 3) SERCA1 y SERCA2 difieren en su perfil de desnaturalización térmica, 4) hay interacción directa de CZL en ambas isoformas de SERCA según el análisis térmico 5) β3-AR está presente tanto en músculo esquelético rápido como en el lento. Esta información nos lleva a considerar que CZL es capaz de penetrar y cruzar las membranas y tener un efecto en el lado interno de

la fibra muscular e interactuar con la SERCA en el SR. Con esto en mente, existe una posible explicación que involucra a la SERCA en la pérdida en producción de fuerza y aumento en la fatiga observada en el músculo esquelético.

6. Conclusión

En conclusión, hemos visto que el Carazolol un agonista β3-adrenoceptor utilizado en la salud y la producción animal tiene un efecto perjudicial sobre la función del músculo esquelético que puede explicarse por una interacción directa con SERCA, una proteína de membrana intracelular a cargo de la relajación muscular. El hallazgo de que Carazolol tiene un fuerte efecto sobre la fatiga del músculo esquelético nos proporciona una herramienta farmacológica para investigar la naturaleza molecular de la resistencia lenta a la fatiga muscular. Además, dado que nuestros hallazgos revelan que el Carazolol está inhibiendo SERCA2 y que también hay evidencia de fatiga en el soleo, estos resultados proporcionan una fuerte evidencia a teorías que explican que la causa fisiológica de la fatiga surge de la modificación de la función de SERCA. Se requiere investigación adicional para explorar esta hipótesis donde el Carazolol podría ser una herramienta potencial. El hecho de que un agonista de β-Adrenoceptores como el Carazolol tenga una acción perjudicial en músculo esquelético pone en tela de juicio el uso de tales fármacos en la industria de producción animal. Las implicaciones del uso del CZL sobre la producción animal y el consumo humano de carne ha de ser considerado.

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