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Intracellular effect of β3-adrenoceptor agonist Carazolol on skeletal muscle, a direct interaction with SERCA

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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 skeletal muscle (SM) proteolysis. Here we sought to better understand the direct effect CZL has on SM. 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 SM and on the mechanical properties of isolated muscle. In isolated SR from SM previously incubated with 0.03 mM CZL, but absent during SR isolation and during SERCA activity determination, the activity was reduced by 45%. 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 was exposed to CZL, inhibition of SERCA occurred in a dose dependent manner. Slow and fast SM T_i of SERCA shifted to a lower temperature in the presence of CZL and a second transition appears at temperatures < 40 °C. In isolated *extensor digitorum longus* (EDL) and *soleus* muscles, CZL reduces the contraction force and increases susceptibility to fatigue. However, recovery force after fatigue in either muscle was higher. Our results suggest that Carazolol penetrates the plasma membrane and interacts with SERCA, thus having an important effect on skeletal muscle function. The inhibition of SERCA may lead to a decrement in SR Ca^{2+}-release promoting further failure in muscle contraction.

1. Introduction

Skeletal muscle (SM) is the most abundant tissue in vertebrates, of which 80% dry weight pertains to protein [1]. In general, SM is divided into two main types. Fast SM is characterized by having glycolytic metabolism; fast contraction-relaxation (c-r) cycles and is sensible to the physiological process of fatigue. Conversely, slow SM has oxidative metabolism, slow c-r cycles and is resistant to fatigue, which can be defined as a reversible decline in force production after prolonged activity [2]. Each muscle type presents an isoform of Ca^{2+}-regulatory protein present in the Sarcoplasmic Reticulum (SR); the Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA), SERCA1 in fast acting twitch muscle and SERCA2 present in slow twitch muscle. Both SERCA isoforms are single unit integrated membrane proteins that mediate the ATP driven intraluminal transport of Ca^{2+} against a concentration gradient [3]. Previous studies have revealed that each isoform is synthesized from two distinct and separate homologous genes [4]. SERCA has the highest affinity for Ca^{2+} removal from cytoplasm and is mainly responsible for setting resting Ca^{2+} concentrations [5]. The removal of Ca^{2+} from the cytoplasm by SERCA leads to muscle relaxation while massive release of SR Ca2+ through the Ryanodine Receptor Ca2+-channel (RyR) causes muscle contraction. Contraction force and fatigue resistance are mechanical properties that are affected by systemic conditions such as nutrition [6], muscle activity [7,8], diseases [9,10], ageing [11,12] and pharmacological effects [13,14]. Skeletal muscle expresses β1 and β2-AR that upon activation increase glycolysis and protein accretion [15,16]. The β3-AR were first known to be expressed in white and brown adipose tissue where upon activation, increase lipolysis [17,18]. The existence of β3-AR in SM has been suggested in other studies through pharmacological studies, where a functional population involved in proteolysis inhibition has been noted [19,20]. Furthermore, direct evidence of β3-AR expression in human SM has been confirmed using specific monoclonal antibodies [21]. The β blocker CZL is well known potent agonist of the human and murine β3-AR [22]. For this, it has been used in human and veterinary medicine as an antihypertensive drug [23,24]. Even though most research involving

Abbreviations: SM, Skeletal Muscle AR, adrenoceptor; BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular calcium concentration; CBD, calcium binding domain; c-r, contraction-relaxation; CZL, carazolol; EDL, *extensor digitorum longus*; P_i, inorganic phosphate; PLB, phospholamban; S.D.M., standard deviation of the mean; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; T_i, transition temperature of inactivation; RyR, Ryanodine Receptor Ca^{2+}-channel

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CZL has been used systemically in animals, its 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.

In the present study, we observed that CZL when incubated in intact SM and washed out before SR isolation, inhibited SERCA activity. Suggesting an effect on Ca^{2+} regulation during c-r cycles through SERCA inhibition. We therefore investigate whether CZL effect on SM function is related to its permeability of Sarcolemma. We used thermal analysis to see if CZL interaction with SERCA is direct. The thermal inactivation analysis of SERCA hydrolytic activity in SERCA1 determined in mainly fast SM and SERCA2 determined in mainly slow SM, indicates a direct effect on the enzyme consistent with the premise that CZL can penetrate the cell. Ultimately, leading to an inhibitory effect on muscle mechanical properties, being a mechanism independent of the β3-AR signalling pathway.

2. Materials and methods

2.1. Animals

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

2.2. Experiments in isolated sarcoplasmic reticulum

2.2.1. Isolation of sarcoplasmic reticulum

Male Wistar rats weighing 280–300 g were euthanised by cervical dislocation on the same day of SR isolation. The EDL and soleus were quickly isolated, which predominantly contain fast and slow twitch fibres respectively. Membranes were obtained from fast and slow skeletal muscle (EDL and soleus muscles). The isolation was performed by differential centrifugation in a discontinuous sucrose gradient as previously described [25,26]. Membrane isolation was performed in the absence of any reducing agent in the buffer. The microsomal fraction was placed in a sucrose gradient of 25%, 27.5% 32% and 35% (w/v). The interface 27.5/32% was identified as Light SR (LSR), 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.

2.2.2. Isolation of SR from intact muscle exposed to extracellular Carazolol

Isolated EDL muscle incubated in Krebs solution with 0.03 mM CZL for 10 min, was placed in the experimental chamber. After incubation, muscle was washed to remove CZL. The treated muscles were used for SR membrane isolation at 4 °C as described in Section 2.2.1.

2.2.3. SERCA hydrolytic activity

Total ATPase activity was measured in isolated LSR by the colorimetric determination of P_i using malachite green as described by Lanzetta [27]. Aliquots of 5 μg protein/ml were incubated in a solution containing in mM; 100 KCl, 5 $MgCl_2$, 5 NaN_3, 20 Tris-malate, 0.1 $CaCl_2$ and 0.34 Mg-ATP (pH 7). The reaction was stopped 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.

2.2.4. Thermal inactivation of ATPase activity

LSR membranes (0.05 mg/ml) were incubated in a solution containing in mM: 100 KCl, 5 $MgCl_2$, 5 NaN_3, 20 Tris-malate and 0.1 $CaCl_2$ (pH 7) in a thermal cycler at a heating rate of 1°C/min as described previously [28,29]. 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 determined, and an inactivation curve was generated. The first derivative of the inactivation curve was used to determine the T_i, which is defined as the temperature recorded for half inactivation. For all inactivation experiments, where the curves are not symmetrical, the T_i does not correspond exactly to half of the curve.

The values for SERCA activity were normalized to those of maximal activity at 30 min of reaction of the control group equal 1. For SERCA inactivation curves the values were normalized to those of maximal activity at 30 min of reaction of the control equal 1.

2.3. Intact muscle experiments

2.3.1. Muscle preparation

Male Wistar rats weighing 280–300 g were euthanised by cervical dislocation, and the EDL and soleus muscles were isolated at room temperature. The isolated muscle was placed into an acrylic chamber that was equipped with platinum electrodes along each side of the chamber wall to allow contact with the Krebs solution as previously described [30,31]. Briefly, we used Krebs solution containing (in mM) 135 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 11 dextrose, 1 $NaPO_4$ dibasic, and 15 $NaHCO_3$ and a gas mixture of 95% O_2 and 5% CO_2 to reach a pH of 7.0. The EDL muscle was fastened by its distal tendon to forceps and by its proximal tendon to a force transducer (FT-03, Grass Medical Instruments, RI, USA). The platinum electrodes were connected in parallel to two stimulators (S88, Grass Medical Instruments, RI, USA).

2.3.2. Stimulation protocol

Single twitch (ST) of 0.6 ms were used to reach the voltage for maximal tension. To obtain the optimal sarcomere length (2.4 μm), the muscles were stretched to the length at which the twitch force was maximal. Fig. 4 shows the schematic representation of the stimulation protocol; the muscles were stretched to the optimum length at which the two Single Twitch (ST) force was maximal (1 Hz and 100 V), followed by six tetanic stimulations (T1 to T6) of 75 Hz for 3 s at 90 V followed by 2 min rest. The calculated force was considered the control maximal tetanic tension and the weight of the muscle. At the end of the protocol, the muscle was rested for 20 min before new stimulation was applied to probe for muscle force recovery (R).

All values are expressed as means ± standard deviation of the mean (SDM). Normalized force was obtained from the maximal force of each group equal 1 (control and in presence of CZL).

2.4. Dot blot assay

Microsomal fractions of EDL and soleus muscles were isolated from male Wistar rats weighting 280–300 g. The isolated microsomal fractions were analysed by dot blot assay. Aliquots of approximately 5 μl 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 min. Signal for dot-blot were developed using Millipore Immobilon system. Using ImageJ 1.6.0 (NIH), the total optical density above local background surrounding was independently determined for each representative dot. The relative optical density was calculated by dividing the densitometry of each sample with the white adipose tissue control.

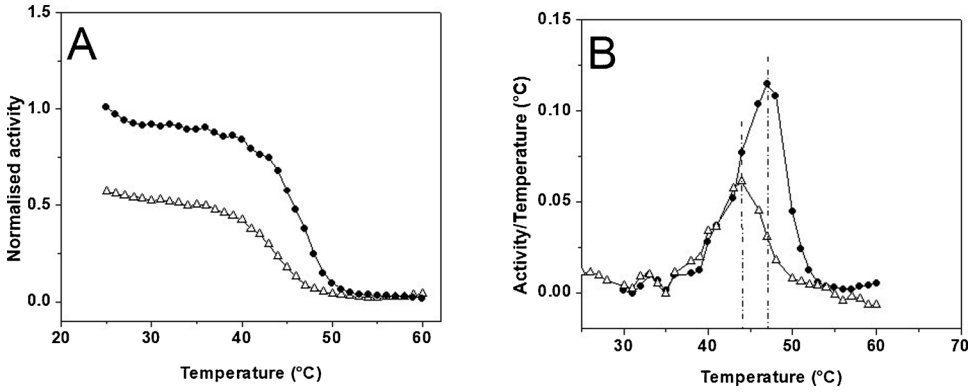

Fig. 1. SERCA1 activity previously incubated with 0.03 mM Carazolol. A) Thermal inactivation curve of: (●) SERCA1 hydrolytic activity control, n = 5 and (△) SERCA1 activity in isolated SR form EDL muscle incubated with 0.03 mM Carazolol prior to SR isolation. B) Invers of the derivative curve of SERCA1 thermal inactivation as a function of increased temperature calculated from Fig. 1A.

3. Results

3.1. Carazolol on SERCA activity

3.1.1. Effect of extracellular applied Carazolol in intact muscle on the isolated SR SERCA

Isolated EDL muscle incubated in Krebs solution with 0.03 mM CZL for 10 min, was placed in the experimental chamber. After incubation, muscle was washed to remove CZL. The treated muscles were used for SR membrane isolation. Fig. 1 shows the effect of muscle incubated with CZL on the SERCA1 hydrolytic activity. Fig. 1A displays the normalised thermal inactivation of SERCA; where activity was 45% lower at 25 °C for the muscle previously incubated with CZL. To better visualize the inactivation temperature (T_i) in these cases, the inverse of the first derivative of the thermal inactivation curves was generated and plotted in Fig. 1B, for control muscle the T_i was 47 °C ± 0.7 and 44 °C for muscle pre-incubated with CZL (n = 5).

3.1.2. Carazolol on SERCA1 from isolated SR of EDL muscle

The effect of CZL on SERCA1 from isolated EDL muscle SR, was dose-dependent. The curves in Fig. 2A reveal the concentration dependent effect of CZL on SERCA1 specific activity, which for control SR corresponds to 20 μmole Pi/mg/min (100%), where 12% ± 1 ($p < 0.1$) of the activity is lost with 0.25 mM CZL with respect to control, while 50% ± 19 ($p < 0.1$) is lost at 0.5 mM (n = 3).

The kinetics of SERCA1 in the absence and presence of 0.25 and 0.5 mM CZL is reduced, at 30 min the activity is inhibited by 24% ± 4 and 37% ± 8 (n = 3) respectively (Fig. 2B). To observe if CZL inhibition was due to a direct interaction with SERCA1 a thermal inactivation analysis was performed. The thermal inactivation curves in Fig. 2C displays the inactivation of SERCA at 30 min of reaction, every minute from 25 to 60 °C, as a function of increasing temperature at a scan rate of 1 °C/min, in the absence and presence of 0.25 and 0.5 mM CZL. Fig. 2D represents the first derivative of the thermal inactivation curves of hydrolytic activity from control SERCA1 with a Ti at 47 °C ± 0.7 (n = 5) ($p < 0.01$). In the presence of CZL two well-defined transitions were observed; in 0.25 mM CZL the Ti was at 45 °C ± 1.5 ($p < 0.02$) and at 32 °C ± 0.1 (n = 3); in 0.5 mM CZL the Ti was at 43 °C ± 0.7 and at 37 °C ± 0.3 (± AEM, n = 2)

3.1.3. Carazolol on SERCA2 from isolated SR of soleus muscle

The effect of CZL on SERCA2 isolated from soleus muscle SR was dose-dependent as well. Fig. 3A shows the concentration dependent effect of CZL on SERCA2 specific activity which for control SR

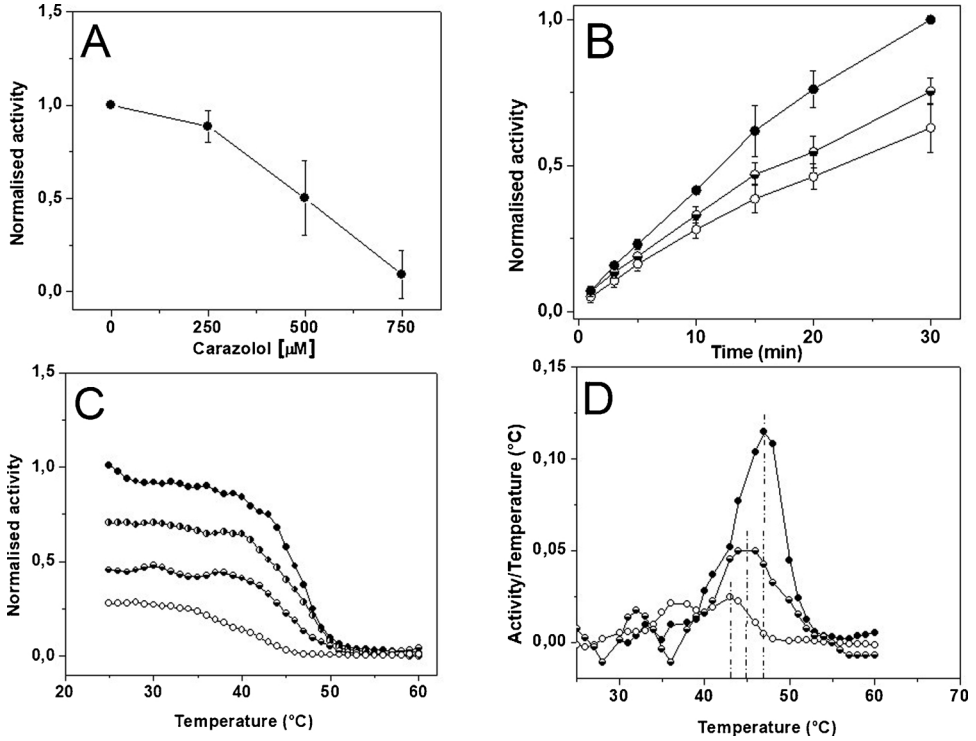

Fig. 2. Effect of Carazolol on SERCA1 from isolated SR of EDL muscle. A) Carazolol concentration effect on specific activity of SERCA1 hydrolytic activity n = 3, (normalised values to those of maximal activity at 1 min reaction). B) Hydrolytic activity of SERCA1 (●) control; n = 3, (◒) 0.25 mM; n = 3 and (○) 0.5 mM Carazolol; n = 3 as a function of time (normalized values to those of maximal activity at 30 min reaction of the control). C) Thermal inactivation curves of SERCA1 hydrolytic activity (●) Control; n = 5, (◑) 0.1 mM; n = 2, (◒) 0.25 mM; n = 3 and (○) 0.5 mM CZL; n = 2, (normalized values to those of maximal activity at 30 min reaction of the unheated control). D) 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 are calculated from the data in Fig. 2C.

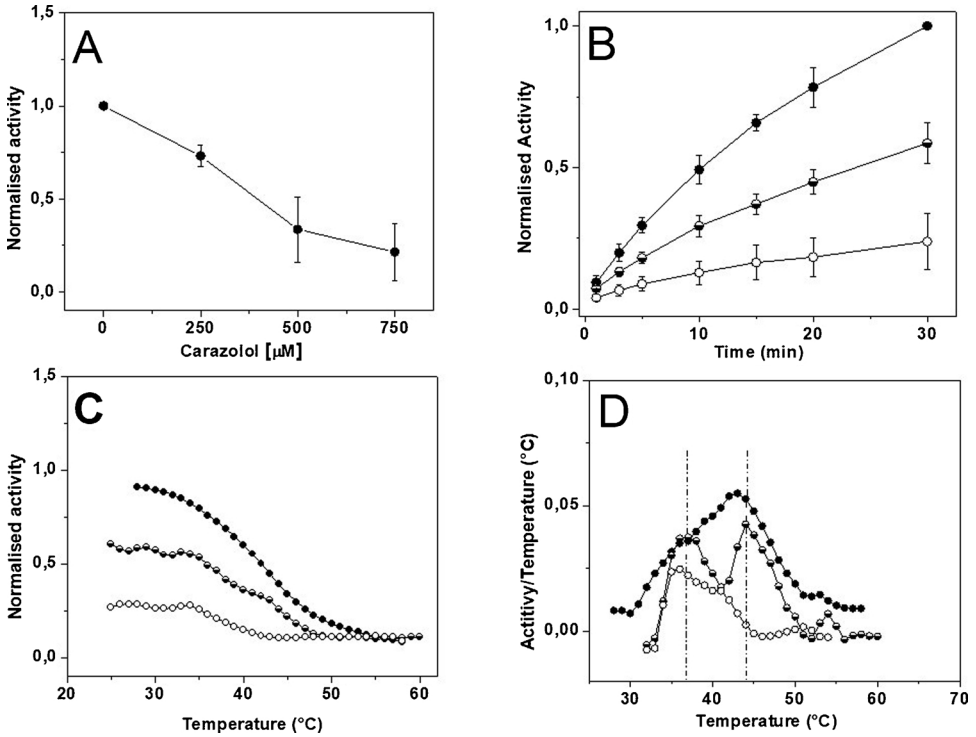

Fig. 3. Effect of Carazolol on SERCA2 from isolated SR of soleus muscle. A) Carazolol concentration effect on SERCA2 hydrolytic activity n = 4 (normalised values to those of maximal activity at 1 min reaction). B) Hydrolytic activity of SERCA2 (●) Control; n = 4, (◒) 0.25 mM; n = 4 and (○) 0.5 mM Carazolol; n = 4, (normalized values to those of maximal activity at 30 min reaction of the control). C) Thermal inactivation curves of SERCA2 hydrolytic activity (●) Control; n = 5, (◒) 0.25 mM; n = 3 and (○) 0.5 mM Carazolol; n = 2, (normalized values to those of maximal activity at 30 min reaction of the unheated control). D) 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 are calculated from the data in Fig. 3C.

corresponds to 10 μmole Pi/mg/min (100%), where 27% ± 5 ($p < 0.02$) of the activity is lost with 0.25 mM, 67% ± 17 (n = 4) ($p < 0.01$) is lost with 0.5 mM CZL. Fig. 3B displays the kinetics of SERCA2 in the absence and presence of 0.25 and 0.5 mM CZL, at 30 min the activity is inhibited by 41% ± 7 and 76% ± 9 (n = 4) respectively. To observe if CZL inhibition was due to a direct interaction with SERCA2 a thermal inactivation analysis was performed. The thermal inactivation curves in Fig. 3C displays the inactivation of SERCA2 in the absence and in the presence of 0.25 and 0.5 mM CZL as a function of increasing temperature at a scan rate of 1 °C/min from 25 to 70 °C. Fig. 3D represents the first derivative of the thermal inactivation curves of hydrolytic activity from SERCA2 with a Ti at 43 °C ± 1.6 (n = 5) ($p < 0.1$). In the presence of CZL two well-defined transitions were observed; with 0.25 mM CZL the Ti was at 44 °C ± 1.3 ($p < 0.01$) and 37 °C ± 1.8 ($p < 0.1$) (n = 4); with 0.5 mM CZL the Ti was at 41 °C ± 0.7 and at 36 °C ± 0.3 (± AEM, n = 2).

3.2. Effect of Carazolol on isolated muscle mechanical activity

Schematic representation of muscle stimulation protocol used to produce fatigue is shown in Fig. 4.

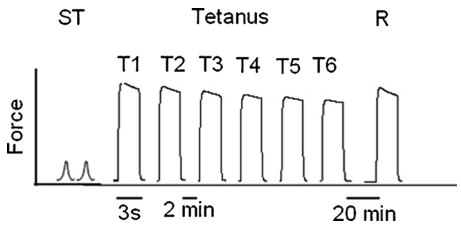

Fig. 4. Schematic representation of the fatigue protocol. Stimulation protocol; two Single twitch (ST) followed by six tetanic stimulations (T1 to T6) were followed by 2 min rest. After T6, a 20 min rest was applied, and a recovery tetanus was then followed (R) with the same parameters of T1 to T6. The detailed protocol is explained under materials and methods (2.3.2.).

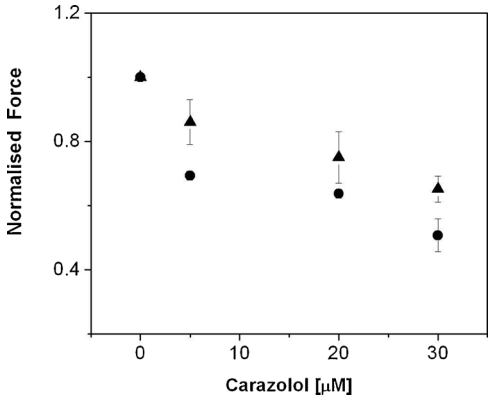

Fig. 5. Muscle force as a function of Carazolol concentration determined after a fatigue protocol. Isolated EDL (●) and Soleus (▲) muscles. The values were normalised to those of maximal force of the control group equal 1 and the maximal force in presence of CZL group equal 1.

3.2.1. Effect of Carazolol concentration on EDL and soleus muscle mechanical activity

Fig. 5 shows the normalised force produced by EDL and soleus muscles in the absence and in the presence of CZL concentrations. At 0.03 mM CZL, EDL muscle force decay 52% ± 7 and in soleus muscle force decay was 36% ± 5 (n = 5).

3.2.2. Effect of Carazolol on muscle fatigue in EDL muscle

Fig. 6 shows the effect of CZL on the tetanic stimulation for EDL muscle. Fig. 6A is a representative experiment expressed as a normalized force where maximal force of each group is equal to 1. Beginning from the first tetanus it was observed that in the presence of CZL fatigue was two times faster. Additionally, decrement in force increases proportionally with subsequent tetanic stimulations. On the sixth tetanic stimulation, the force of muscles incubated with CZL was reduced by 23% ± 7 with respect to the control in the absence of CZL. Fig. 6B is the statistical representation of the experiment presented in Fig. 6A (n = 5).

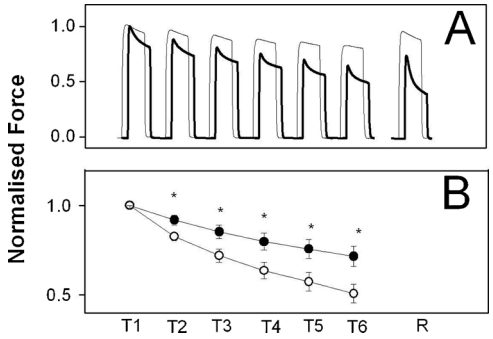

Fig. 6. Effect of Carazolol on EDL muscle fatigue. A) Representative experiment of fatigue and force recovery for control muscle (thin line) and for muscle with 0.03 mM CZL (thick line). B) shows the statistical representation of experiments in panel A, Control (●) and Carazolol (○) n = 5. * P < 0.05. The values were normalised to those of maximal force of the control group equal 1 and the maximal force in presence of CZL group equal 1.

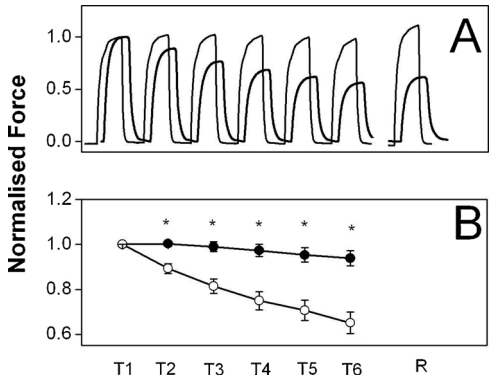

Fig. 7. Effect of Carazolol on Soleus muscle fatigue. A) Representative experiment of fatigue and force recovery for control muscle (thin line) and for muscle with 0.03 mM CZL (thick line). B) Shows the statistical representation of experiments in panel A, Control (●) and Carazolol (○) n = 5. * p < 0.05. The values were normalised to those of maximal force of the control group equal 1 and the maximal force in presence of CZL group equal 1.

3.2.3. Effect of Carazolol on muscle fatigue in soleus muscle

Fig. 7 shows the effect of CZL on the tetanic stimulation for soleus muscle. Fig. 7A is a representative experiment expressed as a normalised force where maximal force of each group is equal to 1. In this muscle, CZL did not have an effect of tetanic force. Therefore, tetanic fatigue is not produced by the CZL. On the sixth tetanic stimulation, the force of muscles incubated with CZL was reduced by 43% ± 8 with respect to the control. Two times more susceptible to loss of force than EDL muscle under the same conditions. Statistical analysis showed that the difference between each tetanus in both muscle types was significant with respect to each point and both curves compared to each other ($p < 0.05$). Fig. 7B is the statistical representation of the experiment presented in Fig. 7A (n = 5).

Since CZL is thought to influence SM through β3-AR, we search for the evidence to probe the presence of β3-AR in isolated Sarcolemma of EDL and soleus muscle.

3.3. β3 adrenoceptors in skeletal muscle

The β3-AR in SM has been determined to be present in SM [19], however as we did not find the experimental evidence, we used a dot blot assay as described in material and methods to provide the experimental evidence to show the presence of β3-AR in fast and slow SM. Fig. 8 show both types of SM contain equal 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 β-AR [18,19].

4. Discussion

The action of CZL on SM, has been observed to cause a decrease in muscle tension and proteolytic activity, bringing about an overall increase in skeletal muscle mass [32]. It is assumed that these effects are mediated exclusively through the specific antagonistic action on β1 and β2-AR and agonist action of β3-AR. However, from the results presented in this study; 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) direct interaction of CZL on both SERCA isoforms as determined by thermal analysis. 4) CZL decrease muscle force in fast and slow SM, 5) and increases fatigue in both SM types 6) β3 AR are present in both fast and slow SM. This data leads us to consider that CZL can cross to the inner side of the SM fibre, penetrates the SR and interact with SERCA. There exists an alternative mechanism that might explain the loss in force and fatigue increase observed in our study, which involves a direct, non-specific effect on SERCA.

4.1. Carazolol effect on SERCA in intact SM

The application of CZL to the external solution of SM would imply that the inhibition of SERCA and muscle mechanical activity we had observed is mediated exclusively through CZL interaction with β-AR. However, when CZL is applied externally to intact SM and washed out before SR isolation, the SERCA hydrolytic activity was inhibited and evaluated by thermal analysis. 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 [26,28,29]. This result signifies that CZL can penetrate and cross the Sarcolemma and access intracellular membranes like SR. CZL labelled with [^{18}F] has been found to cross the hematoencephalic barrier implying its cell membrane diffusibility [33]. Similar results were obtained by PET-studies shown high [^{18}F]-CZL uptake in heart and lung [34]. On the other hand, it has been reported that shortly after intravenous administration of CZL negligible amounts were circulating, but when applied with intramuscular or intra-adipose injection, considerable amount of CZL remain in SM and adipose tissue [35]. Other studies with several hydrophobic compounds have demonstrated that this is not an isolated case. Dihydropyridine receptor antagonists like Verapamil, D-600 and Diltiazem are compounds that have been shown to penetrate the Sarcolemma and interact directly with SERCA [26,36,37].

In rat SM, SR Ca^{2+} uptake variations in fast and slow twitch fibres arise from differences in SERCA1 and SERCA2 isoforms. SERCA1 in fast twitch fibres such as EDL is expressed approximately six times more at the protein level than SERCA2, the isoform predominately expressed in slow twitch fibers such as soleus [38]. Therefore, it is likely to observe dissimilarities when incubated with CZL, especially since the two SERCA isoforms present differences in structure, function and regulation.

4.2. Carazolol effect on SERCA activity in isolated SR

Previous studies have proven that SERCA isoforms present qualitatively similar enzymatic properties [39]. The differences seen in Ca^{2+} transport arise from their expression pattern and in specific regulation in 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 for many species, but apparently absent in rats [40]. The activity of SERCA2 depends on the phosphorylation state of a secondary molecule (PLB) or Sarcolipin (SLN) [41]. Dephosphorylated PLB binds and inhibits Ca^{2+} transport, in its phosphorylated state PLB relieves inhibition, increasing the relaxation rate of

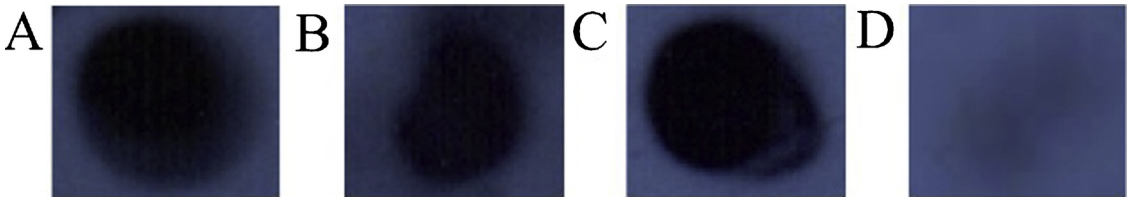

Fig. 8. Dot blot of β-adrenoceptors in skeletal muscle. Representative dot blots of plasma membrane in; A) soleus muscle, B) EDL muscle, C) white adipose tissue D) Bovine Serum Albumin (BSA). White adipose tissue serves as a positive control for β3-AR and BSA as a negative control.

contraction [42]. In this study, both control SERCA isoforms present a similar activity. However, in 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 expressed in its normalized form. A possible interpretation of this data is that CZL is inhibiting the two isoforms by exposing different affinities, since SERCA1 and SERCA2 activity is reduced differently.

4.3. 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 $\triangle Ti = 2\,^{\circ}C$ occurs when CZL 0.25 mM is incubated with SERCA1 and is further reduced with increasing CZL concentration. This indicates that thermal stability is reduced in a concentration dependent manner, which relates with protein conformational changes in SERCA1 with CZL. A comparison of SERCA 1 and SERCA2 from slow SM thermal inactivation profiles has not been evaluated until now. Control SERCA2 presents a $T_i = 43\,^{\circ}C$, further proving that the two isoforms are structurally different as they differ in their thermal inactivation profiles. In both isoforms, it can be said that thermal stability decreases with CZL. Since activity is directly related to structure, the shifts in T_i signify that denaturalization is achieved at lower temperatures when SERCA is 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 from cardiac muscle requires aggregation with PLB in order to become fully active [43]. Although it has been suggested that PLB is absent specifically in rat slow skeletal muscle [44] in our experimental condition SERCA2 behaves as if it is forming oligomers. This suggests that CZL interferes with the association of SERCA2, most likely through a SERCA2 conformational change obstructing the association with PLB or SLN. This new data would then correlate with SERCA2 existing as an oligomer, whose aggregation state is regulated under physiological conditions.

4.4. Effect of Carazolol on the mechanical activity of EDL and soleus muscles

Force production in SM is directly related to intracellular calcium concentration $[Ca^{2+}]_i$ caused by massive Ca^{2+} release from SR. EDL muscle force during tetanic stimulation decreases, implying that the mechanism in charge of Ca^{2+} SR recovery is inhibited during highly repetitive stimulation. Carazolol decreases force in both SM types, being more effective on slow SM, additionally muscle fatigue and force recovery are aggravated in the presence of CZL. As we have shown, SERCA2 function is more prone to CZL than SERCA1. Combined, these results indicate that this difference in sensitivity relates with inhibition of SERCA2. It is well known that fatigue is a physiological phenomenon poorly developed in slow SM [45]. The effects of CZL on fatigue and force recovery in slow muscle imply that fatigue is directly related to SERCA2, as many hypotheses have pointed out for fast SM [46–48]. It can therefore be said that SERCA2 function is more prone to CZL than SERCA1. Thus, fatigue resistance may be related with the oligomerization of SERCA2.

There is a discrepancy on CZL concentration required to reach an effect on SERCA from isolated SR in comparison to the ten times lower concentration to reduce muscle force from isolated muscle. The difference in concentration seen, could be explained by the moment of CZL application. In the case of pre-incubation with CZL, SERCA is in the presence of CZL for 18 h at 4 °C and the activity is measured thereafter. On the other hand, CZL is applied to the isolated SR directly at 25 °C. Since temperature directly affects the conformation of enzymes, we assume that this difference in activity with respect to concentration in these experiments is caused by the fact that SERCA conformation at lower temperature is more susceptible to CZL inhibition, that its effect is maintained even after the isolation protocol is complete. Furthermore, we propose that CZL crosses the plasma membrane barrier but the effect of is not specific. Therefore, more than one cellular component could be targeted and present a synergic effect. Although it has been initially proposed that the pharmacological effect of CZL on SM is through specific activation of β3-AR located in the Sarcolemma, our results strongly imply that CZL effect on SM function can be also explained through SERCA inhibition.

4.5. β3 adrenoceptors (β3-AR) in skeletal muscle

It is well established that β3-AR are present in white and brown adipocytes [49]. Ligand binding studies have shown that membranes from adipose tissue and soleus muscle have identical binding sites for β3-AR as well as a similar population of said receptors in both tissues [20]. Immunoassays confirm that β3-AR is located in SM [21]. We however, have not found the experimental evidence to refer in our discussion. In this study, we confirm the presence of the β3-AR and show that both fast and slow skeletal muscle have similar concentration. Additionally, chronic pharmacological *in vivo* experiments with mice suggest that muscle force increases with β3 agonists, assumed to be a result of β3-AR signalling in SM [50]. 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.

In conclusion, we have seen that CZL a suggested 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. Furthermore, 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 physiological resistance to fatigue.

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