

## ORIGINAL RESEARCH

## Expression of calcium-buffering proteins in rat intrinsic laryngeal muscles

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Calcium, laryngeal muscles, Ncx, Pmca1, Serca, store-operated calcium entry.

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### Introduction

The intrinsic laryngeal muscles (ILM) are highly specialized muscles involved in vital functions that include respiration, phonation, and airway protection (Hinrichsen and

**Abstract**

Intrinsic laryngeal muscles (ILM) are highly specialized muscles involved in phonation and airway protection, with unique properties that allow them to perform extremely rapid contractions and to escape from damage in muscle dystrophy. Due to that, they may differ from limb muscles in several physiological aspects. Because a better ability to handle intracellular calcium has been suggested to explain ILM unique properties, we hypothesized that the profile of the proteins that regulate calcium levels in ILM is different from that in a limb muscle. Calcium-related proteins were analyzed in the ILM, cricothyroid (CT), and *tibialis anterior* (TA) muscles from male Sprague–Dawley rats (8 weeks of age) using quantitative PCR and western blotting. Higher expression of key Ca<sup>2+</sup> regulatory proteins was detected in ILM compared to TA, such as the sarcoplasmic reticulum (SR) Ca<sup>2+</sup>-reuptake proteins (Sercas 1 and 2), the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger, phospholamban, and the Ca<sup>2+</sup>-binding protein calsequestrin. Parvalbumin, calmodulin and the ATPase, Ca<sup>2+</sup>-transporting, and plasma membrane 1 were also expressed at higher levels in ILM compared to TA. The store-operated calcium entry channel molecule was decreased in ILM compared to the limb muscle and the voltage-dependent L-type and ryanodine receptor were expressed at similar levels in ILM and TA. These results show that ILM have a calcium regulation system profile suggestive of a better ability to handle calcium changes in comparison to limb muscles, and this may provide a mechanistic insight for their unique pathophysiological properties.

Dulhunty 1982; DelGaudio et al. 1995; Hoh 2005). These functions are possible due to ILM unique features that include specific fiber-type composition, high speed contraction, and resistance to fatigue (Hinrichsen and Dulhunty 1982; Hoh 2005). Their ability to precisely control

airway caliber during phonation requires specific characteristics reflected in their mechanical properties, innervation pattern (Maranillo et al. 2005; Shinnars et al. 2006; Xu et al. 2009), regenerative capacity, response to age (Kersing and Jennekens 2004; Goding et al. 2005; McLoon et al. 2007), and disease (Marques et al. 2007; Thomas et al. 2008; Smythe 2009). Within the ILM, the posterior cricoarytenoid (PCA) muscle abducts the vocal fold, whereas the thyroarytenoid and cricothyroid (CT) adducts and tenses the vocal fold to aid in phonation and airway protection during swallowing (Hinrichsen and Dulhunty 1982; Hoh 2005). An interesting property of the ILM, in comparison to limb muscles, is their differential involvement in neuromuscular disorders. While ILM are involved early in myasthenia gravis, amyotrophic lateral sclerosis with bulbar involvement, and mitochondrial myopathy (Debain et al. 1968; Mao et al. 2001), they are histologically spared in Duchenne muscle dystrophy (DMD), except for the CT muscle (Marques et al. 2007; Thomas et al. 2008; Smythe 2009).

The ILM play important roles in initiation of and recovery from acute respiratory distress syndrome (ARDS) and critical illnesses, with both swallowing and phonation disability (Nixon et al. 2010). Additionally, vocal muscle paralysis is an important and frequent condition seen in otolaryngology clinics, most frequently caused by lesions to the recurrent laryngeal nerve (Li et al. 2014), although idiopathic etiologies remain common causes of unilateral vocal fold immobility (Rosenthal et al. 2007). Possible mechanisms underlying this differential response of ILM to muscle diseases, compared to limb muscles, may be related to differences regarding their neuromuscular junction structure or satellite cell regenerative potential (Feng et al. 2012). In the *mdx* mouse model of DMD (Bulfield et al. 1984), we have suggested that ILM differ from the affected limb muscles in terms of their ability to handle changes in calcium due to differential levels of calcium-buffering proteins (Ferretti et al. 2009), similarly to what is observed in the extraocular muscles, which are also protected in dystrophy (Khurana et al. 1995; Porter et al. 1998).

In skeletal muscle fibers, intracellular calcium ( $\text{Ca}^{2+}$ ) plays an important role in regulating muscle force production, metabolism, and muscle gene expression (Berchtold et al. 2000; Chin 2010). The sarcoplasmic reticulum (SR) and T-tubule membrane proteins play central roles in calcium regulation. In excitation contraction coupling, depolarization of the plasma membrane leads to the release of  $\text{Ca}^{2+}$  from the SR through ryanodine receptors (RyRs), which are opened via a direct physical interaction with the dihydropyridine receptors (DHPR, CACNA1S), voltage sensors localized in the T-tubules (Melzer et al. 1995; Gailly 2002). At the end of stimulation,  $\text{Ca}^{2+}$  is

pumped back into the SR by sarcoplasmic reticulum  $\text{Ca}^{2+}$  ATPases (SERCA), extruded from the cell by the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX) (Berridge et al. 2003) and buffered by the low-affinity, high capacity  $\text{Ca}^{2+}$ -binding protein calsequestrin (CASQ) (Beard et al. 2004). Other proteins that also play a role in  $\text{Ca}^{2+}$  regulation include the T-system plasma membrane  $\text{Ca}^{2+}$ -ATPase type I (PMCA1) and the store-operated calcium entry (SOCE), which uses the SR  $\text{Ca}^{2+}$  sensor Orai1  $\text{Ca}^{2+}$  channel (Lang et al. 2012). In addition, cytosolic  $\text{Ca}^{2+}$ -binding proteins, such as parvalbumin (PVALB), calmodulin (CaM), and regucalcin (RGN), can modify  $\text{Ca}^{2+}$  transients to ensure  $\text{Ca}^{2+}$  homeostasis (Gailly 2002; Berridge et al. 2003; Beard et al. 2004; Yamaguchi 2005; Lang et al. 2012).

In this study, we evaluated the profile of calcium regulation systems in ILM compared to limb muscle (*tibialis anterior*, TA), in adult rats, using qPCR and Western blotting. We were interested to see whether ILM shows a constitutive calcium-buffering proteins profile that could predict their better capacity to handle calcium changes in comparison to limb muscle and EOM. We found that  $\text{Ca}^{2+}$  reuptake-related proteins of the sarcoplasmic reticulum (*Serca1*, *Serca2*, *Pln*, *Casq1*, and *Casq2*) were expressed at higher levels in ILM in comparison to the limb muscle. ILM showed higher levels of *Orai1* in relation to TA and CaM was 17-fold increased in ILM compared to TA. A similar pattern of gene expression of *Sercas 1* and *2* and calsequestrins was observed in the dystrophin-deficient *mdx* mice compared to the rat. These results provide a mechanistic insight into ILM physiological properties and response to pathophysiological conditions.

## Methods

### Animals

All animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, using protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania School of Medicine (protocol #A3079-01) and by the Committee of the University of Campinas (protocol #1463-1). Male Sprague-Dawley rats ( $n = 20$ ; 8 weeks of age), control C57BL/10ScSnJ (Ctrl,  $n = 10$ ; 8 weeks of age), and *mdx* mice (C57BL/10ScSn-*Dmd*<sup>*mdx*</sup>/J;  $n = 10$ ; 8 weeks of age) obtained from Jackson Laboratory were used in all experiments. The mice and rats were housed according to institutional guidelines and received food and water ad libitum during all experiments.

The muscles studied were the ILM (thyroarythenoid, posterior, and lateral cricoarythenoid), the cricothyroid

(CT) muscle isolated from the other ILM and the *tibialis anterior* (TA) muscle from rats and dystrophic mice. The CT muscle was evaluated separately as it is affected in comparison to the other ILM, which are spared in dystrophin-deficient *mdx* mice (Marques et al. 2007; Thomas et al. 2008; Smythe 2009). We also analyzed the extraocular muscles (EOM: medial, lateral, superior, and inferior rectus), for further comparison of the mechanisms of calcium handling in very fast muscles (Zeiger et al. 2010).

### RNA isolation and SYBR Green-based qPCR

RNA isolation was performed using Trizol reagent (Ambion, Austin, TX) in combination with RNeasy Fibrous Tissue Mini Kit following the RNA protocol as indicated by the manufacturer (Qiagen, Valencia, CA). For SYBR Green qPCR up to 5  $\mu$ g of RNA was reverse transcribed using the Superscript II First Strand Synthesis kit using Oligo(dT) primers according to the manufacturer's instructions. Primers were designed using PrimerExpress 2.0 (Applied Biosystems, Foster City, CA) across exon boundaries (Table 1). qPCR was run on a 7900HT ABI Prism real-time PCR instrument (Applied Biosystems). GAPDH served as reference gene. Fold change and statistical analysis was performed and  $P < 0.05$  considered statistically significant.

### Western blot analysis

Western blot analysis was performed using the NuPage System precast gels as described by the manufacturer (Invitrogen, Carlsbad, CA). Crude whole muscle homogenates were prepared using TNEC lysis buffer (50 mmol/L Tris-HCl pH 8, 150 mmol/L NaCl, 1% Igepal, 2 mmol/L EDTA) containing a complete protease inhibitor cocktail and PhosStop phosphatase inhibitors (Roche, Basel,

Switzerland) (Ferretti et al. 2009). The protein concentration was determined using the DC assay (BioRad). Equal amounts (30  $\mu$ g) of samples were resolved on 4–12% Bis-Tris gels, transferred onto nitrocellulose membranes (Millipore, Billerica, MA) and probed with the following mouse or rabbit antibodies: anti-SERCA 1 (mouse monoclonal Serca1 ATPase, MA3-911, Affinity Bioreagents, Golden, CO); anti-SERCA2 (rabbit polyclonal Serca2 ATPase, ab3625, Abcam, Cambridge, MA); anti-Calsequestrin (recognizes Casq1 and Casq2; rabbit polyclonal, PA1-913, Affinity Bioreagents, Golden, CO); anti-Calmodulin (rabbit monoclonal, MA3-918, Affinity Bioreagents, Golden, CO); anti-CaMkIIB (mouse monoclonal, ab72604, Abcam, Cambridge, MA); anti-ORAI 1 (rabbit polyclonal, ProSci Incorporated, #4281); Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary goat-anti-mouse or goat-anti-rabbit antibodies were conjugated with horseradish peroxidase (Jackson Immuno Research, West Grove, PA). Protein bands were detected with a LAS-3000 Fuji imaging system (Fujifilm, Tokyo, Japan). Equal loading was confirmed after the transfer by Ponceau S staining (Sigma, St. Louis, MO). Bands were quantified densitometrically using Image J software (<http://rsbweb.nih.gov/ij/> National Institutes of Health). Statistical analysis was carried out using Student's t-test, with  $P < 0.05$  considered significant.

## Results

### Increased mRNA expression of Ca<sup>2+</sup>-handling proteins in rat ILM

We determined mRNA levels of genes involved in Ca<sup>2+</sup> homeostasis in ILM's and TA muscles by qPCR. On the basis of a previous report (Zeiger et al. 2010), we

**Table 1.** Primers designed using PrimerExpress 2.0 (Applied Biosystems) across exon boundaries.

Accession number	Gene	Gene Symbol	Forward	Reverse
NM_017290	<i>Atp2a1</i>	<i>Serca1</i>	5'-GCTCGGAACATCTGGAGGGA-3'	5'-GGCACAAGGGCTGGTACTTC-3'
NM_058213.1	<i>Atp2a2</i>	<i>Serca2</i>	5'-GGGAGAACATCTGGCTCGTG-3'	5'-GCGGTACTCCAGTATTG-3'
NM_053311	<i>Atp2b1</i>	<i>Pmca1</i>	5'-GTAGTGGCCGTGATTGTCGC-3'	5'-AGCGTGTCCATGATGAGGTTG-3'
L04684.1	<i>Cacna1s</i>	<i>Cacna1s</i>	5'-CCCCTGTCATGGCTAACCAA-3'	5'-GCCTGGGTTCTGAGGGAAGTC-3'
NM_031969	<i>Calmodulin 1</i>	<i>Calm</i>	5'-AATCCGTGAGGCATTCAGAG-3'	5'-TCTGTTAGCTTTCCCGAGG-3'
XM_001063867	<i>Calsequestrin1</i>	<i>Casq1</i>	5'-GACTTCCCACTGCTGGTCCC-3'	5'-TCCATATGCTGTCCGCATCC-3'
NM_017131	<i>Calsequestrin2</i>	<i>Casq2</i>	5'-GGAGCATCAAAGACCCACCC-3'	5'-TTCTCCGCAAATGCCACAAT-3'
NM_017008	<i>Gapdh</i>	<i>Gapdh</i>	5'-CCATGGAGAAGGCTGGGG-3'	5'-CAAAGTTGTCATGGAT-3'
NM_019268	<i>Ncx1/Slc8a1</i>	<i>Ncx</i>	5'-TTGTCGCTCTTGAACCTCAG-3'	5'-GCTCCGGTGACATTGCCTAT-3'
NM_022499	<i>Parvalbumin</i>	<i>Pvalb</i>	5'-CATTGAGGAGGATGAGCTGGG-3'	5'-CTTGCTCCAGCAGCCATCAG-3'
NM_022707	<i>Phospholamban</i>	<i>Pln</i>	5'-GCTGAGCTCCAGACTTCACA-3'	5'-TTGACAGCAGGCAGCCAAAC-3'
NM_031546	<i>Regucalcin</i>	<i>Rgn</i>	5'-ACGTGACATGTGCCAGGGAT-3'	5'-GCAATTCCTTTGACCCCAAGA-3'
XM_341818	<i>Ryanodine Receptor 1</i>	<i>RyR1</i>	5'-CAAGCGGAAGGTTCTGGACA-3'	5'-TGTGGGCTGTGATCTCCAGAG-3'

separated the genes into five functionally distinct groups of  $\text{Ca}^{2+}$ -related proteins (Table 2). The first group contained genes of the sarcoplasmic, plasma membrane  $\text{Ca}^{2+}$  pumps, and the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger. The second group contained genes that act as regulators of the Serca pumps. The third group contained the main  $\text{Ca}^{2+}$ -binding proteins of the SR. The fourth group of genes contained cytosolic  $\text{Ca}^{2+}$ -binding proteins that buffer elevated intracellular  $\text{Ca}^{2+}$ . The fifth group of genes examined included  $\text{Ca}^{2+}$  channels involved in EC coupling.

The mRNA of eight (*Serca2*, *Casq1*, *Casq2*, *Pln*, *Pvalb*, *CaM*, *Pmca1*, and *Ncx*) of the 12 genes quantified were expressed at significantly higher levels in ILM in comparison with TA (Fig. 1 and Table 2). The highest fold change was detected for the plasma membrane  $\text{Ca}^{2+}$  pumps *Pmca1* and *Ncx* (up to 30-fold increase in ILM vs. TA). *Pln* and *Casq2* (about ninefold increase), followed by *CaM* presented a sixfold increase in ILM versus TA, (Fig. 1 and Table 2). *Pvalb* was increased to a lesser extent (1.8-fold increase in ILM compared to TA; Fig. 1 and Table 2). There were no differences in mRNA expression levels of *Rgn*, *Serca1*, *RyR1*, and *Cacna1s* in ILM compared to TA muscle (Fig. 1 and Table 2).

In the cricothyroid (CT) muscle, most of the quantified mRNAs (7 of 12; 58%) were significantly lower expressed in comparison with other ILM (Fig. 1 and Table 2), with main changes seen in *Pvalb* (twofold decrease in CT vs. ILM), followed by *Serca1* and *Casq2* (about 3.6-fold decrease in CT compared to ILM). *Rgn* was higher expressed in CT than in all the other muscles (Fig. 1 and Table 2). Conversely, the expression of most of the mRNAs in CT (6 of 12; 50%) was comparable to that observed in the limb muscle. *Rgn*, *Pln*, *Pmca1*, *Ncx*, and *CaM* were expressed significantly higher in CT than in TA.

The comparison of EOM with ILM showed that ILM display a similar profile of mRNA expression seen in EOM, for most of the proteins studied, except for *Pmca1* and *Ncx* (higher in ILM than EOM) and *Serca2* (higher in EOM than ILM).

### Increased protein levels of $\text{Ca}^{2+}$ -handling proteins in rat ILM

The relative expression levels of selected proteins involved in  $\text{Ca}^{2+}$  homeostasis were examined using western blots. Figure 2 shows the levels of CASQ1 and CASQ2, SERCA1 and SERCA2, CaM, CaMKII, and Orai1. ILM presented higher levels of CASQ1, SERCA1, SERCA2, CaM, CaMKII, and Orai1 compared to TA (Fig. 2). CASQ2 was higher in TA than in ILM. The levels of the proteins in CT were increased in relation to the levels observed in limb (CASQ1, CASQ2, SERCA1, CaM, CaMKII, and

Orai1) and closer to the levels observed in ILM (CASQ2, SERCA1, and CaM and CaMKII).

### mRNA expression of calsequestrin and Serca in the *mdx* mice

The dystrophic ILM showed increased expression of *Casq1*, *Casq2*, *Serca1*, and *Serca2* compared to the limb dystrophic muscle (Fig. 3). In the *mdx* ILM, gene expression of these SR calcium-related proteins was comparable (*Casq1*, *Serca1*, and *Serca2*) or increased (*Casq2*) in comparison to control ILM. The gene expression of all the proteins (*Casq1*, *Casq2*, *Serca1*, and *Serca2*) was elevated in the affected CT muscle in relation to its respective control.

## Discussion

We found that the  $\text{Ca}^{2+}$  reuptake-related proteins of the sarcoplasmic reticulum, *Serca1* and *Serca2*, the Serca regulator *Pln* and the  $\text{Ca}^{2+}$ -binding protein *Casq1* and *Casq2* were expressed at higher mRNA levels in rat ILM compared to limb muscle. In addition, plasma membrane calcium pumps (*Pmca* and *Ncx*) were significantly increased in rat ILM compared to TA. These results show that rat ILM myofibers display a  $\text{Ca}^{2+}$ -buffering profile that predict a better ability to handle increases in the cytosolic  $\text{Ca}^{2+}$ , in comparison to the limb muscle, which may help protect them from pathophysiological conditions.

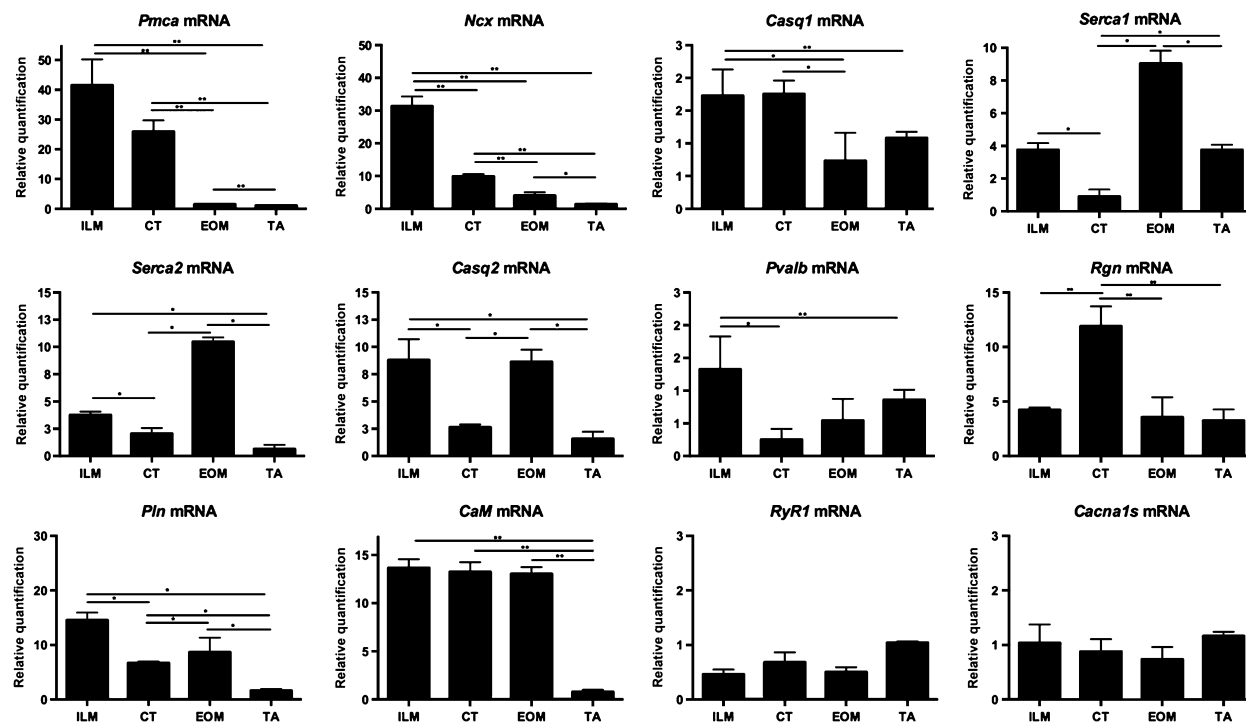
Previously, we and others demonstrated that ILM from the *mdx* mice are spared from dystrophy (Marques et al. 2007; Thomas et al. 2008; Smythe 2009), possibly due to higher levels of the calcium-binding proteins calsequestrin and Serca (Ferretti et al. 2009). In this study, we add further information regarding gene expression of these proteins in dystrophic ILM and found a similar pattern seen in the rat, that is, the expression of these proteins is augmented in ILM (in the dystrophic and in the rat) compared to limb muscle (in the dystrophic and in the rat; except for *Serca1*, which showed comparable expression in rat ILM x TA). The present results suggest that calsequestrin and SERCA expression and levels (Ferretti et al. 2009) profile is a constitutive feature of laryngeal muscles and may help protect ILM myofibers from  $\text{Ca}^{2+}$ -mediated damage, at least in muscular dystrophy. Furthermore, these results suggest that this profile does not necessarily represent an adaptation to the lack of dystrophin in the *mdx* ILM.

*Cacna1s* gene is a voltage-gated calcium channel that encodes the alpha-1S subunit of the DHPR, which is the conducting channel and carries the voltage sensor- and dihydropyridine-binding site (Krasnyi and Ozerniuk 2011). *Cacna1s* has been involved in several biological processes and disorders of skeletal muscles, such as

**Table 2.** Genes encoding Ca<sup>2+</sup>-handling proteins analyzed in intrinsic laryngeal muscles (ILM), cricothyroid (CT), and tibialis anterior (TA) muscles of adult rats. The mRNA levels of these genes were determined by SYBR Green qPCR. Sarcoplasmic reticulum (SR).

Protein category	Protein Function	Gene	ILM versus TA		CT versus TA		ILM versus CT	
			Fold-change	Statistical significance	Fold-change	Statistical significance	Fold-change	Statistical significance
Pumps	SR Ca <sup>2+</sup> -ATPase, fast twitch	<i>Sercaf</i>	1.5 ± 0.43	n.s.	0.7 ± 0.29	<0.05	4.9 ± 1.35	<0.05
	SR Ca <sup>2+</sup> -ATPase, slow twitch	<i>Serca2</i>	1.5 ± 0.40	<0.05	1.2 ± 0.34	n.s.	3.1 ± 0.88	<0.05
	Ca <sup>2+</sup> -ATPase 1, plasma membrane	<i>Pncal</i>	34.7 ± 14.9	<0.05	11.5 ± 2.75	<0.05	2.3 ± 0.53	n.s.
Regulatory proteins	Na <sup>+</sup> /Ca <sup>2+</sup> -exchange, plasma membrane	<i>Ncx</i>	19.8 ± 7.45	<0.05	5.2 ± 1.47	<0.05	3.5 ± 0.45	<0.05
	SR, regulates SERCA	<i>Phospholamban</i>	9.6 ± 0.36	<0.05	4.9 ± 1.02	<0.05	2.2 ± 0.28	<0.05
	cytosol, regulates SERCA	<i>Regucalcin</i>	2.0 ± 0.55	n.s.	5.4 ± 1.1	<0.05	0.4 ± 0.50	<0.05
SR calcium binding proteins	SR, calcium binding protein, skeletal	<i>Calsequestrin 1</i>	1.0 ± 0.25	<0.05	1.2 ± 0.07	n.s.	0.9 ± 0.17	n.s.
	SR, calcium binding protein, skeletal	<i>Calsequestrin 2</i>	9.6 ± 2.61	<0.05	1.5 ± 0.25	n.s.	2.9 ± 0.65	<0.05
	Cytosol, calcium binding protein	<i>Parvalbumin</i>	1.8 ± 10.27	<0.05	0.8 ± 0.08	n.s.	2.1 ± 0.42	<0.05
Calcium channels	Calcium binding protein	<i>Calmodulin</i>	6.5 ± 1.41	<0.05	5.0 ± 1.00	<0.05	2.3 ± 0.74	n.s.
	Calcium channel subunit	<i>Cacna is</i>	0.4 ± 0.08	n.s.	1.1 ± 0.32	n.s.	2.6 ± 0.90	n.s.
	Calcium release channel, skeletal	<i>Ryanodine receptor 1</i>	0.2 ± 0.68	n.s.	0.4 ± 0.04	n.s.	1.5 ± 0.37	n.s.

Mean fold changes ± SEM from three independent muscle samples, each sample containing muscles pooled from three or four different rats were calculated and analyzed using REST 2005. P < 0.05 was considered statistically significant.

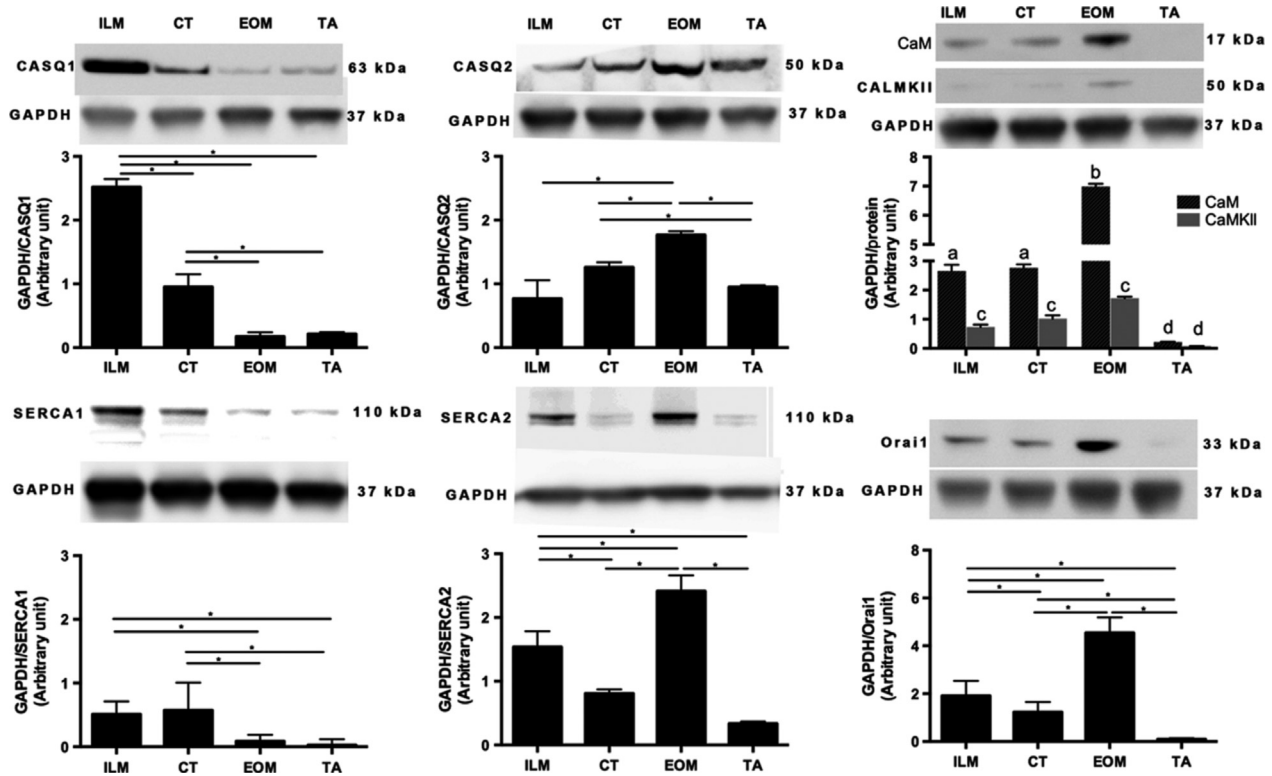


**Figure 1.** Relative mRNA levels of genes encoding  $\text{Ca}^{2+}$ -handling proteins in intrinsic laryngeal muscles (ILM), cricothyroid (CT) muscle, extraocular muscle (EOM), and *tibialis anterior* (TA) muscle. The genes studied were  $\text{Ca}^{2+}$ -ATPase 1, plasma membrane (*Pmca1*),  $\text{Na}^+/\text{Ca}^{2+}$ -exchange, plasma membrane (*Ncx*),  $\text{Ca}^{2+}$ -ATPase slow twitch (*Serca2*), calsequestrin 2 (*Casq2*), phospholamban (*Pln*), calmodulin (*CaM*), calsequestrin 1 (*Casq1*),  $\text{Ca}^{2+}$ -ATPase, fast twitch (*Serca1*), parvalbumin (*Pvalb*), regucalcin (*Rgn*), ryanodine receptor 1 (*RyR1*), and calcium channel subunit (*Cacna1s*). The relative mRNA levels of genes encoding  $\text{Ca}^{2+}$ -handling proteins were determined by SYBR Green qPCR in muscle tissue. Quantifications from three independent muscle samples, each sample containing muscles pooled from three or four different rats. Mean fold changes  $\pm$  SEM of three independent samples each were calculated and statistically analyzed using REST 2005 and  $*P < 0.05$ ,  $**P < 0.01$  was considered statistically significant.

malignant hyperthermia and muscle channelopathies (MacLennan and Zvaritch 2011; Burge and Hanna 2012). The RyR1 channel complex is mainly involved in promoting the release of  $\text{Ca}^{2+}$  from the SR, dampening calcium oscillation that is required for continued myofiber contractions (Bellinger et al. 2008). The abundant SR in ILM (compared to limb muscle) (Hinrichsen and Dulhunty 1982) could implicate in more releasable sites and consequently higher levels of *Cacna1s* and *RyR1* in ILM than in TA muscle. However, these two  $\text{Ca}^{2+}$ -release proteins were expressed in similar levels in rat ILM and TA. One possibility to explain this finding could be related to differences in the activity status of these proteins, rather than their expression, between ILM and TA muscles. Overall, the present findings (i.e., higher levels of SR  $\text{Ca}^{2+}$ -reuptake  $\times$  similar levels of SR  $\text{Ca}^{2+}$ -release proteins in ILM vs. TA) raise the possibility that  $\text{Ca}^{2+}$ -reuptake mechanisms are more important than  $\text{Ca}^{2+}$ -release mechanisms for ILM myofibers to maintain their functional properties, but further studies would be necessary to elucidate this point.

Besides the SR, another  $\text{Ca}^{2+}$  influx pathway is the store-operated  $\text{Ca}^{2+}$  entry (SOCE), which is stimulated by reduction in intracellular  $\text{Ca}^{2+}$  stores (Putney 1990). The molecular components of the SOCE include STIM1, an endoplasmic/sarcoplasmic reticulum (ER/SR)  $\text{Ca}^{2+}$  sensor (Liou et al. 2005; Zhang et al. 2005) that translocates from the ER/SR membrane to regions close to the plasma membrane following depletion of the intracellular  $\text{Ca}^{2+}$  stores (Wu et al. 2006). This movement of STIM1 activates Orai1, a pore-forming unit that allows permeation of  $\text{Ca}^{2+}$  through the plasma membrane into the cytosol (Prakriya et al. 2006; Wang et al. 2010). Orai1 was significantly increased in rat ILM in comparison to TA muscle. Orai1 protein abundance is under regulation of a wide variety of cellular mechanisms, including hormones (Lang et al. 2012). ILM are under the modulation of several hormones that can influence their fiber types (Hoh 2005), which could explain the differences between ILM and TA muscle observed here regarding their Orai1 profile.

In this study, we also explored the levels of the cytoplasmic-buffering proteins Pvalb and CaM. The genes for

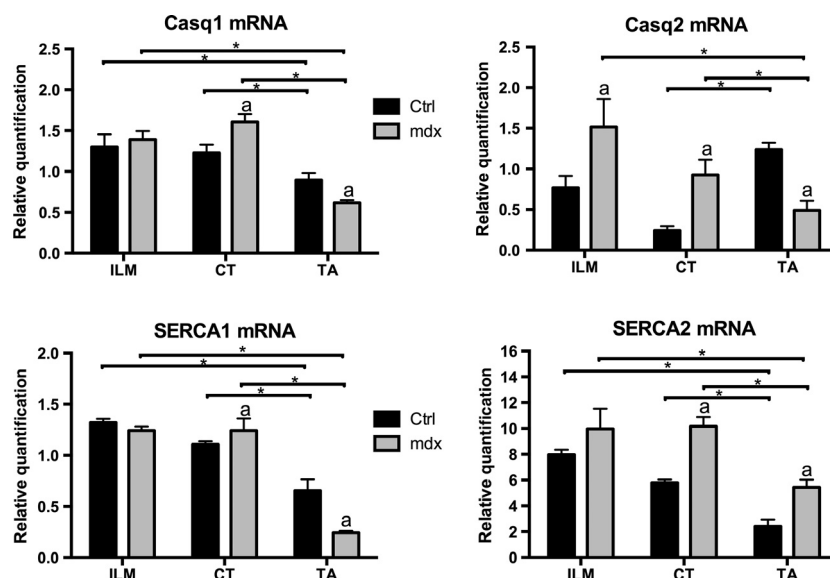


**Figure 2.** Protein levels of sarcoplasmic reticulum  $\text{Ca}^{2+}$ -handling proteins in intrinsic laryngeal muscles (ILM), cricothyroid (CT) muscle, extraocular muscles (EOM), and *tibialis anterior* (TA) muscle. Western blot analysis showing relative abundance of indicated proteins: calsequestrins 1 and 2 (CASQ1 and CASQ2), Sercas 1 and 2 (SERCA1 and SERCA2), calmodulin (CaM), calmodulin kinase II (CaMKII), and Orai1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for protein loading. Western blot transfer and nonspecific changes in protein levels. The molecular weight, expressed in kDa, for each protein is indicated. Quantifications from three independent muscle samples, each sample containing muscles pooled from three or four different rats. Asterisks and different letter combination indicate statistical significance (\* $P < 0.05$  and ab, ac, ad, bc, or bd  $P < 0.05$ , respectively). In ILM, CASQ 1 was more abundant than CASQ2 compared with TA. SERCA1 was less than SERCA2 in ILM and higher compared with TA. CT and the other ILM showed similar levels of the proteins studied.

these proteins were significantly increased in rat ILM compared to TA. It is known that fast contracting fibers, such as ILM myofibers (Hoh 2005), contain high concentrations of *Pvalb* (Heizmann et al. 1982). However, *CaM* mRNA expression in ILM was almost 10 times higher than *Pvalb* and threefold increased in ILM compared to TA. This finding suggests that, in ILM, calmodulin may represent an important mechanism of cytosolic  $\text{Ca}^{2+}$  buffering. Furthermore, similarly in EOM,  $\text{Ca}^{2+}$ -CaM signaling mechanisms in ILM may ensure the production of other molecules, such as utrophin, to help protect against dystrophy (Porter et al. 1998).

Cricothyroid (CT), the vocal cord tensor, resembles limb muscles in MyHC composition and fiber-type properties (Hoh 2005). Considering their calcium-buffering profile, we observed that rat CT shares some similarities with the limb muscle (*Serca2*, *Ncx*, *Casq2*, and *Pvalb*), but also shows similarities with others ILM (*Serca1*, *Casq1*, *RyR1*, *CaM*, and *Orai1*). Previously, using

histopathological methods, we demonstrated that the dystrophic CT muscle was affected significantly, showing an increased percentage of central nucleated fibers when compared to the other ILM (Marques et al. 2007). We suggested that decreased expression of calcium-binding proteins could explain the susceptibility of CT to dystrophy (Ferretti et al. 2009). However, in this study, we observed augmented gene expression of all the main SR calcium-related proteins, including *Serca2*, in the *mdx* CT, suggesting that other calcium-related proteins and/or mechanisms may be involved in CT dystrophy. We observed a dramatic increase in *Rgn* in rat CT compared to other ILM and TA. *Rgn* is an important cytosolic calcium-handling protein, involved in several intracellular signaling mechanisms (Yamaguchi 2005) and may be involved in the functional differences among CT and other ILM previously reported (Hinrichsen and Dulhunty 1982). This study also points out that CT and ILM present a distinct expression profile for wild-type rats and



**Figure 3.** Relative mRNA levels of genes encoding  $\text{Ca}^{2+}$ -handling proteins in control (Ctrl) and dystrophic (*mdx*) mice in intrinsic laryngeal muscles (ILM), cricothyroid (CT) muscle, extraocular muscle (EOM), and *tibialis anterior* (TA) muscle. The genes studied were  $\text{Ca}^{2+}$ -ATPase slow twitch (*Serca2*), fast twitch (*Serca1*), calsequestrin 2 (*Casq2*), and calsequestrin 1 (*Casq1*). The relative mRNA levels of genes encoding  $\text{Ca}^{2+}$ -handling proteins were determined by SYBR Green qPCR in muscle tissue. Mean fold changes  $\pm$  SEM of three independent samples each were calculated and statistically analyzed using REST 2005. Each independent muscle sample contained a pool of muscles from 3–4 mice. Asterisks indicate statistical significance ( $*P < 0.05$ ). Letter “a” was considered statistically significant ( $P < 0.05$ ) compared with Ctrl.

mice regarding *Serca*s and *Casq2*. A possible explanation for this finding could be related to differences in the rate of respiration due to distinct metabolic rates in rats compared to mice or distinct vocal repertoire (Holy and Guo 2005; Riede 2014). Differences in the contraction times of laryngeal and CT muscles among different species have also been reported, possibly due to differences in SR volume and the content of parvalbumin, a  $\text{Ca}^{2+}$ -binding protein (Hoh 2005).

Higher levels of Pln, CaMKII, and CaM in rat ILM may play a role in regulating *Serca* activity (O'Brien et al. 1993; Morita et al. 2009), through similar mechanisms as those described for the EOM (Simmerman et al. 1986; Traaseth et al. 2008; Zeiger et al. 2010). In this study, we also add information about the SOCE component *Orai1* in ILM and EOM. We observed that *Orai1* showed a similar profile in ILM versus EOM, that is, significantly elevated compared to the limb muscle. Interestingly, EOM showed significantly higher levels of these proteins in relation to ILM. Overall, despite both muscles groups (ILM and EOM) display very fast contraction cycles, they have minimal differences in their  $\text{Ca}^{2+}$ -buffering profile that may reflect differences in their functional properties, such as the fine control of airway caliber by ILM and fine control of eye movement, by the EOM.

Vocal fatigue, a symptom of a voice disorder that occurs as a consequence of prolonged voice use mainly

reported in singing, teaching and acting professionals (Welham and Maclagan 2003), may contribute to the development of other laryngeal pathologies (Kostyk and Rochet 1998). While the etiology of vocal fatigue is poorly understood, neuromuscular junction fatigue, changes in vocal fold viscosity and reduced blood circulation seem to be involved (Welham and Maclagan 2003). Fatigue in skeletal muscles seems to be related to a decrease in the amplitude of the myoplasmic  $\text{Ca}^{2+}$  transient which is thought to result from changes in the function of SR-calcium-related proteins (Fitts 2011). Recently, the plasma membrane transporter NCX has been suggested to play a role in the resistance to muscle fatigability in slow twitch muscles (Michel et al. 2014). We observed that most (58%) of the calcium-related proteins mRNAs in the rat were significantly lower expressed in the CT muscle in comparison with other ILM, including the NCX transporter. Therefore, although there are no evidences from in vivo studies in humans, it may be that the human CT muscle is less resistant to fatigue compared to other ILM. Overall, it seems reasonable to suggest that alterations in calcium homeostasis due to changes in calcium-related proteins may help explain, at least in part, the decreases in ILM resistance to fatigability in certain pathologies and this may be of relevance to develop new therapeutic strategies to vocal muscle fatigue or even to other laryngeal disturbances.



## Conclusions

This study demonstrates that rat ILM present a constitutive  $\text{Ca}^{2+}$ -buffering profile that predicts their better ability to handle calcium changes in comparison to limb muscles. This profile is in agreement with their function as very fast muscles with a well-developed capacity for prolonged work. Other mechanisms may help explain the differential functional and pathophysiological responses of ILM versus limb muscles. For instance, it would be interesting to study other important SR calcium-related proteins, such as sarcalumenin (Jiao *et al.*, 2012) and sarcolipin (Rossi and Dirksen 2006), which are major  $\text{Ca}^{2+}$ -binding and SERCA regulators (Dowling *et al.* 2004). Nevertheless, this study suggests that mechanisms involved in the prompt sequestering of  $\text{Ca}^{2+}$  (sarcoplasmic reticulum  $\text{Ca}^{2+}$ -reuptake proteins, plasma membrane pumps, and cytosolic  $\text{Ca}^{2+}$ -buffering proteins) are particularly elevated in ILM, indicating their importance for ILM myofiber function and protection against disease. Furthermore, differential levels of Orail in rat ILM and EOM over the limb muscle suggests a role for SOCE in ILM/EOM functional properties and signaling mechanisms.

## Conflict of Interest

None declared.

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