Brazilian Journal of Medical and Biological Research (2009) 42: 21-28
ISSN 0100-879X Review

The inhibitory role of sympathetic nervous system in the Ca^{2+}-dependent proteolysis of skeletal muscle

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Mammalian cells contain several proteolytic systems to carry out the degradative processes and complex regulatory mechanisms to prevent excessive protein breakdown. Among these systems, the Ca^{2+}-activated proteolytic system involves the cysteine proteases denoted calpains, and their inhibitor, calpastatin. Despite the rapid progress in molecular research on calpains and calpastatin, the physiological role and regulatory mechanisms of these proteins remain obscure. Interest in the adrenergic effect on Ca^{2+}-dependent proteolysis has been stimulated by the finding that the administration of β_2-agonists induces muscle hypertrophy and prevents the loss of muscle mass in a variety of pathologic conditions in which calpains are activated. This review summarizes evidence indicating that the sympathetic nervous system produces anabolic, protein-sparing effects on skeletal muscle protein metabolism. Studies are reviewed, which indicate that epinephrine secreted by the adrenal medulla and norepinephrine released from adrenergic terminals have inhibitory effects on Ca^{2+}-dependent protein degradation, mainly in oxidative muscles, by increasing calpastatin levels. Evidence is also presented that this antiproteolytic effect, which occurs under both basal conditions and in stress situations, seems to be mediated by β_2- and β_3-adrenoceptors and cAMP-dependent pathways. The understanding of the precise mechanisms by which catecholamines promote muscle anabolic effects may have therapeutic value for the treatment of muscle-wasting conditions and may enhance muscle growth in farm species for economic and nutritional purposes.

Key words: Epinephrine; Norepinephrine; Calpastatin; Calpain; Skeletal muscle

Presented at the IV Miguel R. Covian Symposium, Ribeirão Preto, SP, Brazil, May 23-25, 2008.

Research supported by FAPESP (#04/02674-0 and #06/0697-3) and CNPq (#305847/06-6 and #300440/05-7).

Received August 1, 2008. Accepted December 4, 2008

Introduction

The sympathetic nervous system (SNS) utilizes two major chemical signaling molecules: epinephrine (adrenaline) that is secreted directly into the blood from the adrenal medulla, and norepinephrine (noradrenaline) that is the major neurotransmitter produced and released from peripheral sympathetic neurons, which are distributed widely to different tissues and are associated with blood vessels. Although it is well known that skeletal muscles receive a cholinergic innervation from the motoneuron, evidence indicates that adrenergic nerve terminals make close contact with striated muscle fibers in mammals (1). However, the physiological role of this innervation is far from being well established.

It is well known that most of the metabolic actions of the SNS in several tissues are exerted through a β-adrenoceptor-mediated increase in intracellular cyclic adenosine

monophosphate (cAMP) and subsequent activation of cAMP-dependent protein kinase (PKA). The intracellular concentration of cAMP in cells is determined via the balance between cAMP production by adenylyl cyclase, and cAMP degradation by phosphodiesterases (PDEs). Skeletal muscle contains numerous isoforms of PDE, including PDE4, PDE7 and PDE8 (2). However, PDE4 appears to contribute to the majority of cAMP degradation in this tissue (3).

Historically, the physiological role of SNS is related to a "fight or flight" response that prepares an organism to cope with an emergency. Catecholamines decrease the uptake of glucose in peripheral tissues, partly through an inhibition of insulin secretion, stimulate glycogenolysis (4), increase the diet-induced thermogenesis in brown adipose tissue (5), stimulate the substrate oxidation in skeletal muscle (4), and have a well-known stimulatory effect on white adipose tissue lipolysis (6). They also have a marked effect on protein metabolism in skeletal muscle. Numerous studies have shown that β_2-adrenergic agonists, such as clenbuterol and cimaterol, induce hypertrophy of skeletal muscle in livestock and humans (7). β-agonist-induced hypertrophy seems to be specific for striated muscle, since the smooth muscle of the gut, liver, and kidney (8) do not increase in size in response to these agents. It has also been reported that treatment with β_2-adrenergic agonists reduces muscle wasting in different catabolic situations including tumors, atrophy denervation, sepsis, muscular dystrophy, hindlimb suspension, hyperthyroidism, burning, and diabetes (7,9,10). However, the biochemical mechanisms of these effects of β_2-adrenergic agonists are not completely understood.

Skeletal muscle protein mass depends on the balance between synthesis and degradation. Even a small decrease in synthesis or a small acceleration of degradation, if sustained, can result in a marked loss of mass in the organism (11,12). The main intracellular proteolytic systems in the skeletal muscles are the lysosomal, the Ca^{2+}-dependent, and the ubiquitin-proteasome system. During the last decade, we have been studying the mechanisms through which the rates of these different proteolytic components are regulated by hormonal, nutritional and neural factors. This review will focus on recent studies of the influence of the SNS on Ca^{2+}-dependent proteolysis, as well as on the mechanisms involved.

The role of the sympathetic nervous system on muscle protein breakdown

Knowledge of the regulatory factors involved in the control of the rates of protein degradation is important for understanding the mechanisms by which hormones, neurotransmitters, nutrition, growth promoting agents, and environmental and genetic factors influence growth. In order to investigate the physiological role of catecholamines in the control of protein breakdown in skeletal muscles, we have been using the chemical sympathectomy experimental model in which rats are treated with guanethidine for a few days (13). Guanethidine was chosen because it has been shown to produce a selective blockade of norepinephrine release from peripheral nerves without affecting central adrenergic neurons (14). Guanethidine treatment induces a drastic 90% reduction in norepinephrine content of soleus muscles and a 40-80% reduction in plasma levels of norepinephrine and epinephrine (13). After 1 and 2 days of guanethidine treatment, there was a significant 20% increase in the rate of proteolysis in incubated soleus muscles. Because this early rise in the proteolytic rate occurred without a concomitant change in the plasma levels of other hormones, it was interpreted to be a direct consequence of the depletion of muscle norepinephrine and/or of the reduction in plasma catecholamine concentration induced by guanethidine treatment. Also, the acute increase in proteolysis after adrenergic blockade suggested the existence of an inhibitory adrenergic tonus in oxidative skeletal muscles, which restrains proteolysis (13). In agreement with this suggestion, we have found that both epinephrine and norepinephrine, added *in vitro*, reduce the rate of protein degradation in normal rat soleus and extensor digitorum longus (EDL) by approximately 15-20% (15). This view is consistent with the finding that infusion of epinephrine in muscle microdialysis experiments (16), in perfused rat hindquarters (17) and in humans (18) induces a rapid and similar 20% decrease in protein degradation.

Recently, we have investigated the role of the SNS in the control of protein degradation in skeletal muscles from rats with streptozotocin (STZ)-induced diabetes (19). It has been shown that chemical sympathectomy by guanethidine further increased the high rate of protein degradation in soleus and EDL muscles during the early stages of diabetes in rats (1 and 3 days after STZ). Although guanethidine treatment induced a decrease in both plasma epinephrine and muscle norepinephrine, the additional increase in the proteolysis observed in muscles from sympathectomized diabetic rats was probably a consequence of the reduction in plasma epinephrine concentration (19). It has been found that the acute reduction in rat plasma catecholamines produced by surgical removal of the adrenal medulla, with no change in muscle norepinephrine content, is accompanied by a transient increase in the rate of skeletal muscle proteolysis (20). It has also been shown

that the infusion of epinephrine in humans and animals induces a rapid decrease in the activity (21) and gene expression (22) of enzymes involved in muscle protein breakdown. The fact that the effects of sympathectomy were obtained in a condition of an acute catabolic state, like diabetes, supports our previous hypothesis based on experiments with chemical sympathectomy of normal rats, that the SNS exerts an acute effect on skeletal muscle protein metabolism, reducing proteolysis. This anabolic effect of the SNS can be interpreted as a mechanism to spare muscle protein in a situation of catabolism. In fact, the drastic reduction of the catecholamine levels in diabetic-sympathectomized rats promoted an additional fall in the body weight of these animals compared to the diabetic group (19).

The finding that soleus was more sensitive than EDL to the catabolic effect of sympathectomy in diabetic rats (19) is consistent with our previous demonstration (15) that soleus is more sensitive than EDL to the antiproteolytic effect of epinephrine *in vitro*. This differential sensitivity may be explained by differences in the density of β-adrenoceptors and levels of PKA, which have been shown to be higher in rat skeletal muscles rich in type I fibers (e.g., soleus) than in muscles containing mainly type II fibers (e.g., EDL) (23,24). That the adrenergic inhibitory effects on skeletal muscle proteolysis are mediated by β_2-adrenoceptors had already been suggested by the *in vivo* hypertrophic effect of the β_2-adrenergic agonists on skeletal muscle of different species and by the suppression of the clenbuterol-induced hypertrophy of rat gastrocnemius muscles by oral administration of ICI 118,551, a selective β_2-adrenoceptor antagonist (25). Recent experiments from our laboratory strongly support this hypothesis by demonstrating directly in isolated skeletal muscle the mediation by β_2-adrenoceptors. Thus, the antiproteolytic effect of epinephrine in soleus and EDL was completely suppressed by propranolol and by ICI 118,551 (13). Clenbuterol *in vitro* induced a dose-dependent inhibition of proteolysis that was also prevented by ICI 118,551 in both muscles (20). Numerous studies have shown that β_3-adrenergic receptors are also present in skeletal muscle (26,27). The presence in skeletal muscle of other types of β-receptors, different from β_1- and β_2-adrenoceptors, was initially surmised from the observation by Reeds et al. (28) that propranolol blocked the clenbuterol-induced decrease in body fat, but was not capable of reverting the increase in rat skeletal muscle growth promoted by the β_2-agonist. This finding suggested the existence of β-adrenoceptors-mediated responses clearly distinct from those mediated by β_1- and β_2-adrenoceptors in muscle. The presence of functional β_3-adrenergic receptors in muscle has been suggested by the metabolic effects of specific agonists *in vitro* (29) and by the detection of the β_3-adrenoceptor protein and its mRNA in human (26) and rat (27) skeletal muscle. The physiological role of muscle β_3-adrenoceptor, however, remains poorly understood. We have previously shown that CL-316,243, a selective β_3-adrenoceptor agonist, exerts an inhibitory action on proteolysis in rat-isolated soleus muscle. The inhibition of proteolysis induced by the addition of CL-316,243 to the incubation medium of soleus muscles from fed and fasted rats was completely prevented by SR-59230A, a selective β_3-antagonist, but was not affected by selective β_1- or β_2-antagonists (30). Furthermore, we have demonstrated that the inhibitory effect of epinephrine on muscle protein degradation can also be prevented by SR-59230A, suggesting that this adrenergic effect may be mediated by β_3-adrenoceptors (30). It was interesting to note that, unlike soleus, the overall rates of proteolysis observed in fast-twitch muscles, such as EDL, from fed and fasted rats was not affected by CL-316,243. Taken together, these results suggest that epinephrine inhibits proteolysis by activating β_2- and/or β_3-adrenoceptors in oxidative muscles such as soleus (20,30). Further experiments are needed to determine the relative sensitivity of the different muscle fiber types to the β_3-adrenergic agonist-mediated effects on proteolysis.

Mechanism of adrenergic inhibition of the Ca^{2+}-dependent proteolysis

The intracellular Ca^{2+}-dependent proteolytic system contains at least two ubiquitous enzymes: μ- (low Ca^{2+}-requiring form) and m-calpain (high Ca^{2+}-requiring form). Calpains do not degrade proteins to amino acids or even to small peptides (31,32). In fact, calpains initiate myofibrillar protein degradation by disassembling the outer layer of proteins from the myofibril and releasing them as myofilaments. Although the proteasome, the proteolytic enzymatic complex of the ubiquitin-proteasome system, can degrade the majority of intracellular proteins, it cannot degrade myofibrillar proteins until they have been removed from the myofibril by calpains. Therefore, it appears that both calpains and the proteasome are responsible for myofibrillar protein turnover. Calpains tend to be concentrated in the Z band of the sarcomere (33), the site where disassembly begins (34). Since skeletal muscle cells contain sufficient calpain to destroy all Z bands in these cells in 5-10 min, it is likely that in physiological conditions calpain activity must be inactive most of the time (32). Calpain activity is regulated by a variety of factors, including Ca^{2+}, phospholipids, a 30-kDa small calpain subunit, and calpastatin, a widely distributed calpain-specific endoge-

nous inhibitor (35). It is generally thought that the calpain-calpastatin system plays a major role in muscle protein degradation only when cytosolic Ca^{2+} homeostasis is altered. Recent findings showing that calpastatin overexpression results in skeletal muscle hypertrophy (36) and protects mice against atrophy (37) provide evidence that calpastatin is also involved in the control of normal skeletal muscle protein turnover. However, the physiological role of neural and peripheral signals implicated in the control of calpain and calpastatin activity in *in vivo* conditions is still unknown.

In previous studies, we have demonstrated that the increase in overall proteolysis observed after 2 days of chemical (13) and surgical sympathectomy (20) is accompanied by a parallel increase in the activity of the Ca^{2+}-dependent system, suggesting that the restraining effect of the adrenergic tonus on skeletal muscle proteolysis is exerted, at least in part, by keeping the Ca^{2+}-dependent pathway inhibited. In agreement with these results, the inhibitory *in vitro* effect of clenbuterol and CL-316,243 on the rate of overall proteolysis in isolated skeletal muscle from normal rats was shown to be associated with a reduction in the activity of the Ca^{2+}-dependent proteolysis, an effect that was prevented by selective β_2- and β_3-antagonists (20,30).

In view of the antiproteolytic effect of the SNS in skeletal muscles from normal rats, recent studies were undertaken in our laboratory to examine the changes in the rate of protein synthesis and activity of the different proteolytic systems, which occur in oxidative muscles from rats submitted to 9 days of chronic intermittent hypoxia (CIH; 6% O_2 for 40 s at 9-min intervals; 8 h/day). Studies have shown that rats submitted to CIH exhibited a higher level of basal sympathetic activity, which, in turn, increased arterial pressure (38,39). In addition to the lower rates of protein synthesis observed in soleus muscles from rats exposed to CIH, we also found a reduction in the rates of proteolysis (Fabio TL, Gonçalves DA, Lira EC, Zanon NM, Bonagamba LG, Zoccal DB, Kettelhut IC, Machado BH, Navegantes LCC, unpublished data). The CIH-induced inhibition of muscle proteolysis in rats was probably a direct consequence of the muscle sympathoexcitation and can be interpreted as an adaptive response of protein metabolism that helps to prevent excessive loss of skeletal muscle mass during prolonged hypoxic stress. Maintenance of protein content is especially essential for red, oxidative muscles, such as soleus, which serve the important function of sustaining most of the postural work of the animal. The decrease in the overall proteolysis in soleus muscles from rats exposed to CIH was accompanied by ~50% decrease in the activity of muscle Ca^{2+}-dependent proteolytic pathway, with no changes in the activities of the lysosomal and ubiquitin-proteasome systems. There is a growing body of evidence that the activity and gene expression of calpastatin are increased after β_2-adrenergic agonists treatment (40-42), leading to the suggestion that this is the mechanism of the inhibitory effect of catecholamines on the Ca^{2+}-dependent proteolysis. This hypothesis has been reinforced by the findings that CIH induces an increase in calpastatin levels in soleus muscles from rats. Similar results have been observed in soleus from rats treated with clenbuterol for 3 days (Gonçalves DA, Baviera AM, Lira EC, Zanon NM, Kettelhut IC, Navegantes LC, unpublished data) and in muscles from humans after acute exercise (43). Because catecholamines and β-agonists activate PKA and may act via cAMP responsive elements (CRE) in calpastatin promoter regions (44), it has been proposed that calpastatin is a target for this kinase. In agreement with this hypothesis, it has been shown that calpastatin is phosphorylated by PKA (45) and its maximal inhibitory activity against m-calpain is enhanced by phosphorylation in rat skeletal muscle *in vitro* (46). Moreover, recent evidence indicates that the calpastatin gene promoter is activated in rat muscle L6G8 cells transfected with dibutyryl-cAMP or forskolin (47), suggesting that both the calpastatin gene promoter and protein are targets for PKA activity. The inhibition of Ca^{2+}-dependent proteolysis by nonhydrolyzable cAMP analogs and the activation of β_2-adrenergic receptor *in vitro* inhibit proteolysis in skeletal muscle from normal rats (20) and chicks (48) are also consistent with the results of the studies cited above.

These findings indicate that cAMP signaling may play an important role in preventing proteolysis in physiological conditions and raise the possibility of using drugs that induce an increase in the intracellular concentrations of cAMP, as the cAMP-PDE inhibitors, to prevent muscular atrophy in several pathological situations. A nonselective PDE inhibitor (pentoxifylline; PTX) or two different selective PDE4 inhibitors (cilomilast and rolipram) have been administered to mice undergoing muscle atrophy, in order to reduce skeletal muscle cAMP degradation, and thus increase cAMP-mediated muscle hypertrophy (49,50). The selective PDE4 inhibitor rolipram was shown to inhibit protein degradation in muscles from normal rats (Lira EC, Gonçalves DA, Baviera AM, Zanon NM, Kettelhut IC, Navegantes LC, unpublished data) and to prevent the muscle wasting and weakness associated with sciatic nerve resection and limb casting (49). Treatment with PTX induced a clear reduction in the high rates of muscle protein degradation in tumor-bearing rats (51) and during sepsis (52). In another study from our laboratory, Lira et al. (53) perfused skeletal muscles from normal and septic rats

with isobutylmethylxanthine, a nonselective PDE inhibitor, and demonstrated that this compound increased muscle cAMP levels and reduced muscle protein catabolism by inhibiting proteolysis. More recently, Baviera et al. (54) also demonstrated that the *in vivo* and *in vitro* treatment with PTX increased muscle cAMP levels and induced a decrease in the increased rate of total protein degradation in EDL muscles from diabetic animals through a clear reduction in the activity of the Ca^{2+}-dependent and ubiquitin-proteasome-dependent proteolytic systems, two degradative processes well known to be activated in the acute phase of experimental diabetes (55). The antiproteolytic effect of PTX *in vitro* was inhibited by H89, a PKA inhibitor, further supporting the idea that activation of the cAMP cascade via a PKA-dependent pathway is one of the regulatory mechanism(s) to prevent excessive skeletal muscle protein breakdown. Although the PKA signaling pathway is the most commonly studied β-adrenoceptor effector in skeletal muscle (7), other studies have shown that the production of cAMP by epinephrine in skeletal muscle (56) may activate a novel protein termed EPAC (exchange protein directly activated by cAMP). Therefore, the possibility of PKA-independent signaling pathways in regulating proteolysis cannot be ruled out.

Figure 1 illustrates the possible mechanisms by which Ca^{2+}-dependent proteolysis could be regulated by the SNS *in vivo*. Catecholamines bind directly to β_2- and β_3-adrenoceptors located in the sarcolemma, stimulate adenylate cyclase activity, and consequently increase cAMP, which stimulates PKA in skeletal muscles. This kinase could increase calpastatin activity by direct phosphorylation and/or by increasing the levels of calpastatin mRNA by

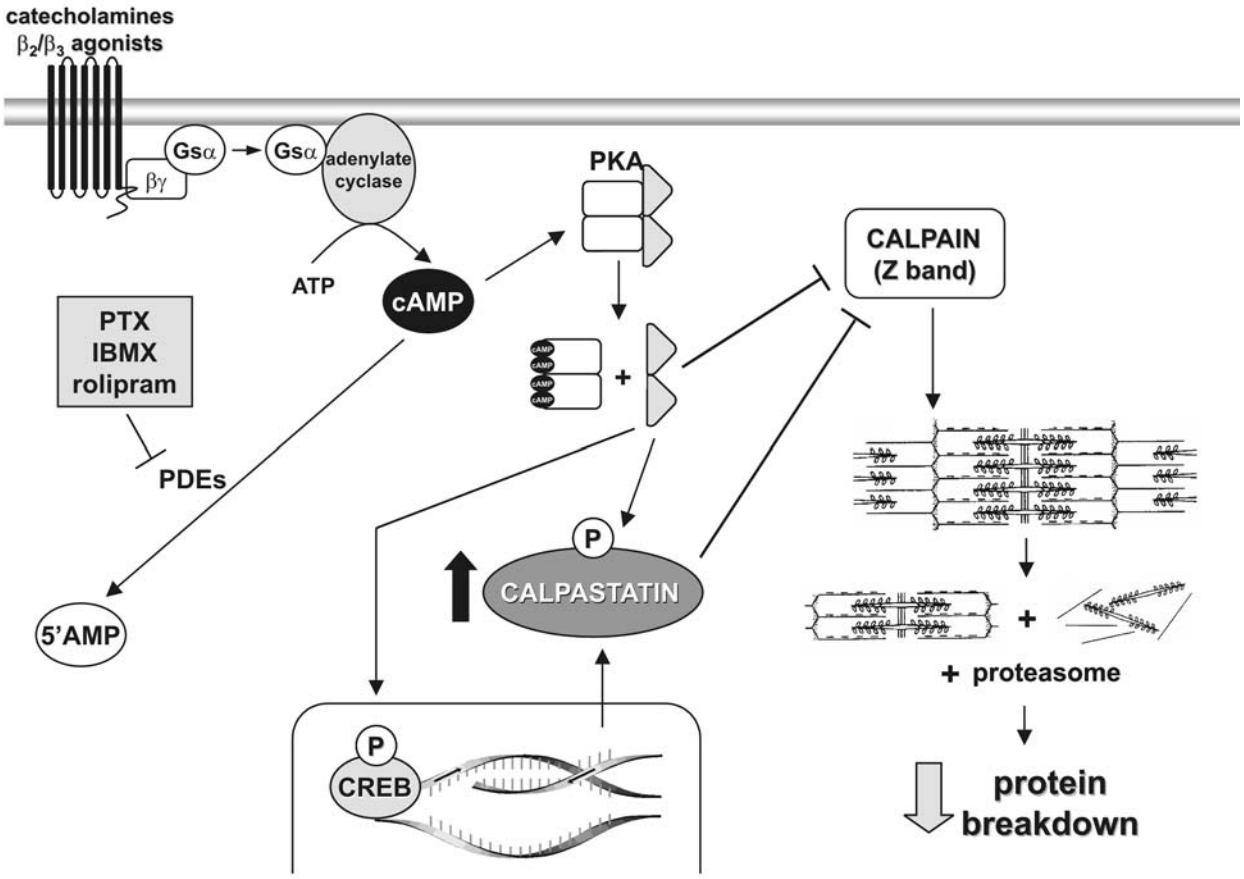

Figure 1. Mechanisms in the inhibition of the Ca^{2+}-dependent proteolysis in skeletal muscle by catecholamines and β-agonists. Gsα = stimulatory alpha subunit of guanine nucleotide-binding regulatory protein; βγ = beta-gamma subunit of guanine nucleotide-binding regulatory protein; ATP = adenosine triphosphate; cAMP = cyclic adenosine monophosphate; 5'AMP = 5'adenosine monophosphate; PTX = pentoxifylline; IBMX = isobutylmethylxanthine; PDEs = phosphodiesterases; PKA = cAMP-dependent protein kinase; CREB = cAMP responsive element binding protein.

enhanced gene transcription through phosphorylation of the cAMP responsive element binding protein (CREB) and activation of CRE, which could keep the calpain activity inhibited. Alternatively, calpain itself might be inhibited by catecholamines through PKA-induced phosphorylation. In fact, evidence in fibroblasts indicates that m-calpain can be directly phosphorylated and inhibited by PKA (57). More recently, it has been demonstrated that β_2-adrenergic agonist treatment attenuates muscle atrophy induced by hindlimb unweighting or atrophy denervation through inhibitory effects on ubiquitin-proteasome system (58,59). Thus, it could be speculated that proteasome and/or the process of protein ubiquitination is also regulated by PKA and/or EPAC.

Conclusion

In contrast to its catabolic effects on carbohydrate and lipid metabolism, the SNS, through adrenal medulla-secreted catecholamines, and norepinephrine released by adrenergic terminals exerts anabolic action on skeletal muscle protein metabolism. In addition to the intrinsic regulation of calpain by the Ca^{2+}-requirement for the enzyme activity, and by levels, activity and isoform types of calpastatin, catecholamines may contribute to maintain muscle Ca^{2+}-dependent proteolysis inhibited through a β_2-adrenoceptor/cAMP-mediated phosphorylation of components of the calpain system, including CREB and calpastatin. In oxidative muscles, such as soleus, at least part of these effects can also be mediated by β_3-adrenoceptors. This antiproteolytic effect seems to be important for the preservation of muscle structure and function, mainly in oxidative postural muscles in both basal conditions and during stress situations. The anabolic actions of synthetic catecholamines and/or cAMP-phosphodiesterase inhibitors may also have therapeutic value in the treatment of muscle-wasting conditions, such as diabetes and sepsis, and may enhance muscle growth in farm animals for economic and nutritional purposes. This emphasizes the necessity of further studies to clarify the molecular mechanisms involved with the adrenergic control of protein metabolism in skeletal muscle.

Acknowledgments

We are indebted to Neusa M. Zanon and Maria Antonieta R. Garófalo (Departamento de Fisiologia), Elza Aparecida Filippin and Victor Diaz Galbán (Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil) for technical assistance.

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