Protein oxidation, tyrosine nitration, and inactivation of sarcoplasmic reticulum Ca^{2+}-ATPase in low-frequency stimulated rabbit muscle

Bert M. Klebl, Ahmed T. Ayoub, Dirk Pette*

Fakultät für Biologie, Universität Konstanz, D-78457 Constance, Germany

Abstract Sustained contractile activity by chronic low-frequency stimulation in rabbit fast-twitch muscle causes a partial (40–50%) inactivation of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase and, with prolonged stimulation, a SERCA1a to SERCA2a transition. To investigate the underlying mechanism of the inactivation which precedes the isoform transition, we analyzed SR from 4-day stimulated muscles for Ca^{2+}-ATPase activity, lipid peroxidation, SH and carbonyl groups, and nitrotyrosine. At unaltered SH group and malondialdehyde contents, carbonyl groups were elevated 50% in the SR from stimulated muscles. Immunoblotting with anti-dinitrophenyl and anti-nitrotyrosine antibodies revealed strong labeling of the Ca^{2+}-ATPase, suggesting the inactivation of the enzyme to result from protein oxidation and peroxynitrite-mediated tyrosine nitration.

Key words: Ca^{2+}-ATPase; Chronic low-frequency stimulation; Inactivation; Nitrotyrosine; Peroxynitrite; Protein oxidation

1. Introduction

Using the model of chronic low-frequency stimulation (CLFS), we have shown that sustained contractile activity of rabbit fast-twitch muscles causes, in addition to a SERCA1a to SERCA2a transition after prolonged stimulation periods [1], a reduction in Ca^{2+} uptake by the sarcoplasmic reticulum (SR) and a reduced catalytic activity of the SR Ca^{2+}-ATPase [1–5]. The reduction of both Ca^{2+} uptake and catalytic activity manifests itself during the first hours and days, reaching maxima after 2–4 days of CLFS. Reduced ATP-, ADP- and phosphate-dependent phosphoprotein formation, decreased labeling by fluorescein isothiocyanate, and reduced ATP binding point to an alteration of the nucleotide binding and phosphorylation sites of the enzyme [3,5]. Moreover, a reduced accessibility to tryptic cleavage suggests a structural change of the Ca^{2+}-ATPase in the stimulated muscle [4].

Free radical formation has been discussed as a major source of work-induced muscle injury [6]. Based on in vitro and in vivo studies, enhanced lipid peroxidation, increased protein oxidation, and reduced SH group content have been suggested to cause inactivation of the SR Ca^{2+}-ATPase [6–16].

We were interested in investigating the role of free radical attack in the inactivation of the SR Ca^{2+}-ATPase in low-frequency stimulated muscle. Low-frequency stimulation is a standardized model for sustained contractile activity and has been shown to elicit pronounced muscle fatigue [17]. The CLFS-induced inactivation of the SR Ca^{2+}-ATPase, therefore, might also provide a link to impaired Ca^{2+} dynamics in muscle fatigue [18–20]. In the present study, rabbit tibialis anterior and extensor digitorum longus muscles were exposed to CLFS for 4 days in order to attain maximum Ca^{2+}-ATPase inactivation [3]. To investigate whether stimulation-induced changes are stable after cessation of stimulation, analyses were performed on muscles obtained immediately or 4 h after cessation of CLFS. Crude SR preparations from stimulated and contralateral muscles were analyzed for Ca^{2+}-ATPase activity, lipid peroxidation, total sulfhydryl group content, 2,4-dinitrophenylhydrazine (DNPH)-reactive carbonyls, tryptophan, tyrosine and bityrosine fluorescence. In view of the enhanced nitric oxide production in working muscle [21,22] and its reaction with superoxide generating the strong oxidant peroxynitrite, we were also interested in the detection of nitrotyrosine, a major product of peroxynitrite attack on protein tyrosine residues [23,24].

2. Materials and methods

2.1. Animals, low-frequency stimulation

Adult male New Zealand White rabbits were subjected to CLFS (10 Hz) using the 12 h/day stimulation protocol (1 h on, 1 h off) as previously described [3]. The rabbits were exposed to CLFS for 4 days. One group of animals (0-h group, $n = 6$) was killed immediately after cessation of stimulation, and a second group was killed 4 h after cessation of stimulation (4-h group, $n = 7$). Left (stimulated) and right (unstimulated control) extensor digitorum longus and tibialis anterior muscles were quickly excised and processed for isolation of crude SR according to [5].

2.2. Protein determination

Three different assays were used, the micro-BCA (bicinchoninic acid) protein assay (Pierce), the Bio-Rad protein assay according to Bradford [25], and the assay according to Lowry et al. [26].

2.3. Ca^{2+}-ATPase activity

The ATP-hydrolyzing activity was determined using both the phosphate liberation assay [15] and the coupled optical test [27]. Ca^{2+}-dependent ATPase activity was determined either by subtracting the reaction rate in the absence of Ca^{2+} or by inhibition with 10 μM cyclopiazonic acid (Sigma), a specific inhibitor of the SR Ca^{2+}-ATPase [28]. The two assays yielded nearly identical results.

2.4. Determination of malondialdehyde

The malondialdehyde content was photometrically determined at 532 nm [29]. 1,1,3,3-Tetramethoxypropane (Aldrich) was used for calibration.

2.5. Gel electrophoresis and immunoblotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done as previously described [4]. Gels were stained with Coomassie blue or transferred to nitrocellulose membranes. Blots were covered with the specific antibodies and developed using alkaline phosphatase-coupled primary or secondary antibodies and CSPD (Boehringer Mannheim) as a substrate. Carbonyl groups were visualized with an anti-dinitrophenyl antibody coupled to alkaline phosphatase (Sigma, 1:5000 dilution). Nitrotyrosine residues were detected by a rabbit polyclonal anti-nitrotyrosine antibody (Upstate Biotechnology, 1:100 dilution). The primary antibody-antigen complex was visualized by chemilumi-

*Corresponding author. Fax: (49) (7531) 883940.
E-mail: dirk.pette@uni-konstanz.de

PII S0014-5793(98)00053-2

nescence after incubation with an anti-rabbit antibody coupled to alkaline phosphatase (1:30 000 dilution) and CSPD as substrate.

2.6. Sulfhydryl group content

The amount of SH groups was estimated according to [30]. 50–300 μg of crude microsomes were incubated in 2.5 ml of incubation medium and after 5 min at room temperature the absorbance was measured at 412 nm. Using a molar extinction coefficient of $\varepsilon = 13\,600\ M^{-1}\ cm^{-1}$ and assuming that Ca^{2+}-ATPase represents 70% of the total microsomal protein in the 4-day stimulated and contralateral muscles [3,5,31], the results are expressed as sulfhydryl groups per 1.5×10^5 g of protein [32].

2.7. Tryptophan, tyrosine and bityrosine fluorescence

15 μg crude microsomes were processed for fluorometric measurements according to [33]. After digestion of the microsomes with proteinase K (Boehringer Mannheim) at 37°C, the samples were precipitated on ice with 1.6 M perchloric acid (PCA). After centrifugation for 15 min at $3000 \times g$ and 4°C, the supernatants were neutralized with 2 N KOH and kept on ice for 1 h. Precipitates were removed by centrifugation at $3000 \times g$ and 4°C. 1 ml of the supernatant was added to 1 ml 0.2 M HEPES/KOH (pH 9.0) and tyrosine fluorescence was determined at 278 nm and 303 nm for excitation and emission, respectively. Tryptophan fluorescence was determined at 290 nm and 345 nm, and bityrosine fluorescence at 315 nm and 420 nm.

2.8. DNPH-reactive carbonyls

The spectrophotometric carbonyl assay for the assessment of protein oxidation was conducted according to [34]. In brief, 1 mg of crude microsomes was incubated for 30 min at room temperature in 0.5 ml of 10 mM DNPH (Sigma) in 2 M HCl. Derivatization was stopped by the addition of 0.5 ml of 20% (w/v) trichloroacetic acid and protein was pelleted by centrifugation for 3 min at $11\,000 \times g$. The pellets were washed three times with 1 ml ethanol/ethyl acetate (1:1). After the last centrifugation, the protein was solubilized in 1 ml 6 M guanidine hydrochloride in 20 mM potassium phosphate (pH 2.3). Insoluble material was removed by centrifugation and maximal absorbance of the supernatant was determined in the range of 360–390 nm. The carbonyl content was calculated using a molar extinction coefficient of $\varepsilon = 22\,000\ M^{-1}\ cm^{-1}$. Carbonyl group-containing proteins were visualized after gel electrophoresis by immunoblotting as described above (see Section 2.5).

3. Results

3.1. Ca^{2+}-ATPase

The catalytic activity of the SR Ca^{2+}-ATPase was 45–50% reduced in 4-day stimulated muscles (Table 1). Both the phosphate liberation assay and the coupled optical test (data not shown) yielded nearly identical values. No significant changes existed between Ca^{2+}-ATPase activities in microsomal preparations from the 0-h and 4-h groups. The reason for studying muscles 4 h after cessation of stimulation was to verify whether or not the stimulation-induced changes were stable during the early phase of recovery.

According to SDS-PAGE (results not shown), Ca^{2+}-ATPase existed as monomer and dimer in both control and stimulated samples. In agreement with previous findings [3,5,31], SR preparations from the 4-day stimulated and contralateral control muscles were obtained in the same yields.

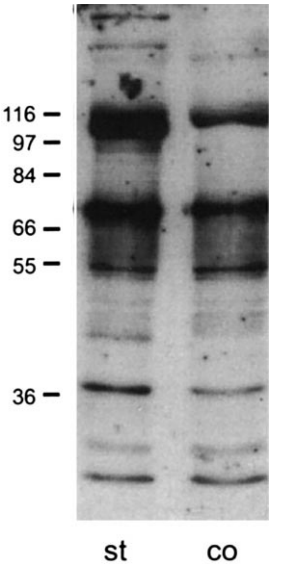

Fig. 1. Carbonyl content of contralateral and stimulated muscle microsomes. 15 μg of crude microsomes from control and stimulated muscles were derivatized as outlined in Section 2. After stopping the reaction and neutralization, the samples were separated by SDS-PAGE on adjacent lanes. The gel was blotted onto a nitrocellulose membrane and carbonyl groups were visualized by reaction with an anti-dinitrophenyl antibody coupled to alkaline phosphatase (1:5000 dilution) using CSPD as a substrate. Abbreviations: co, contralateral muscle; st, 4-day stimulated muscle. Molecular mass markers are indicated in kDa.

3.2. Effects of stimulation on protein determination and specific amino acids

An unexpected finding was the observation that protein determinations by three different assays displayed conspicuous differences when crude SR preparations from control and stimulated muscles were compared. Values obtained by the Lowry and BCA assays regularly yielded elevated protein contents of the stimulated samples. Thus, the possibility existed that protein contents of these preparations were either overestimated by the Lowry and BCA assays or underestimated by the Bradford method. Since tryptophan, tyrosine, and cysteine are reactive groups in the BCA and Lowry assays, and these amino acid residues might have been modified by the action of free radicals [12,13,35], fluorometric analyses were performed to examine whether tyrosine and tryptophan were modified in the preparations from stimulated muscle. However, no significant differences existed between control

Table 1
Ca^{2+}-ATPase activity in crude sarcoplasmic reticulum preparations from contralateral and 4-day stimulated EDL and TA muscles of rabbit

	Ca^{2+}-ATPase activity (μmol/min/mg)		
	contralateral	stimulated	% of control
0-h group	0.583 ± 0.003	0.258 ± 0.021	44.2
4-h group	0.447 ± 0.064	0.223 ± 0.050	49.9

In one group (0-h), animals ($n = 3$) were killed immediately after cessation of stimulation. In a second group (4-h), animals ($n = 3$) were killed 4 h after cessation of stimulation. Ca^{2+}-ATPase activity was determined by the phosphate assay [15]. Values are means ± S.D. from triplicate measurements on each sample. Protein was determined according to Bradford [25].

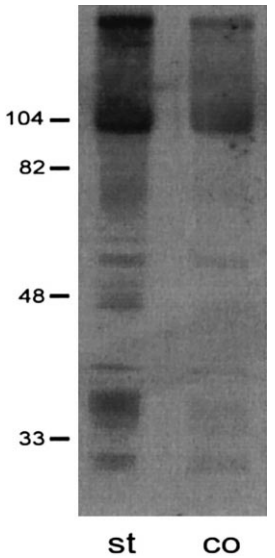

Fig. 2. Protein tyrosine nitration of microsomes from stimulated and contralateral tibialis anterior muscles. 40 μg of crude microsomes from stimulated and contralateral muscles were applied for electrophoretic analysis onto a 10% polyacrylamide gel. After electrophoretic separation, the gel was blotted onto a nitrocellulose membrane. Nitrotyrosine residues were detected with an anti-nitrotyrosine antibody (1:100 dilution). The primary antibody-antigen complex was visualized by an anti-rabbit antibody coupled to alkaline phosphatase (1:30 000 dilution) and CSPD as a substrate. Abbreviations: co, contralateral muscle; st, 4-day stimulated muscle. Molecular mass markers are indicated in kDa.

and stimulated muscles. In order to exclude protein determination artifacts due to amino acid modification, all further analyses were referred to protein data obtained by the Bradford assay [25]. This Coomassie blue-based method depends primarily on the protein hydrophobicity and, therefore, should be less susceptible to interference with experimentally induced putative amino acid modifications [36].

3.3. Lipid peroxidation, sulfhydryl groups, protein oxidation

As assessed by the thiobarbituric acid assay, lipid peroxidation was not elevated in the stimulated muscles. The malondialdehyde content amounted to 1.36 ± 0.18 and 1.19 ± 0.16 per mg microsomal protein in contralateral and stimulated muscles, respectively.

The determination of sulfhydryl groups in crude microsomal preparations did not reveal differences between contralateral and stimulated muscles. We calculated 23.13 ± 2.16 and 22.17 ± 2.69 sulfhydryl groups per 1.5×10^5 g microsomal protein as determined by the Bradford assay in contralateral and stimulated muscles, respectively. The value for normal (contralateral) muscle was in good agreement with published data (e.g. [37]), thus providing independent proof for the feasibility of the Bradford assay in the present study.

Protein oxidation as determined by DNPH-reactive carbonyls was significantly elevated in the stimulated muscles (Table 2). The 0-h and 4-h muscles displayed increases of 53% and 34% in DNPH-reactive groups, respectively. These observations were further verified by immunochemical detection of DNPH-reactive carbonyls in electrophoretically separated microsomal proteins. As shown in Fig. 1, the amount of carbonyl groups was markedly increased in the stimulated muscle. In addition, the band corresponding to the Ca^{2+}-ATPase monomer displayed a stronger intensity than in the control sample.

3.4. Nitration of protein tyrosine

For detection of protein nitrotyrosyl residues, microsomes from control and 4-day stimulated muscles were electrophoretically separated, blotted to nitrocellulose membranes, and exposed to a specific nitrotyrosine antibody [23]. As illustrated by a typical result in Fig. 2, microsomes from stimulated muscle generally displayed a higher reactivity than the microsomal preparation from the corresponding control. Furthermore, the 105 kDa Ca^{2+}-ATPase displayed the highest signal intensity in the chemiluminescent detection of nitrated tyrosine.

4. Discussion

Previous work from our laboratory has shown that sustained contractile activity as imposed by CLFS leads to a partial inactivation of the SR Ca^{2+}-ATPase in fast-twitch muscles of rabbit and rat [1–5,38]. The decrease in specific Ca^{2+}-ATPase activity starts a few hours after the onset of stimulation and reaches a maximum after a few days. It thus precedes the isoform switch of the SR Ca^{2+}-ATPase, i.e. downregulation of the fast SERCA1a and upregulation of the slow SERCA2a with prolonged stimulation [1,39,40]. According to previous studies, the reduced Ca^{2+}-ATPase activity correlates with structural changes of the enzyme [4,31]. Several in vitro studies as well as the present results point to a modification by free radical attack. An in vitro study on isolated SR pointed to singlet oxygen as the active agent in reducing Ca^{2+}-transport ATPase activity [12]. Another in vitro study suggested a direct attack of hydroxyl radicals ($^{\bullet}OH$) on the ATP binding site [15]. Support was gained from the finding that ATP had a protective effect. Recently, Viner et al. [16,41] reported on in vitro effects of peroxyl radicals ($ROO^{\bullet}$) on SR preparations from rabbit fast-twitch muscle. They observed an inhibition of the Ca^{2+}-dependent ATPase activity which correlated with the loss of its monomeric form and the appearance of higher molecular mass species. Oligomerization of the enzyme was thought to result from oxidant-induced bityrosine cross-linking.

As judged from electrophoretic analyses under reducing conditions, our results do not provide evidence for the in vivo formation of oligomeric Ca^{2+}-ATPase. The failure to

Table 2
Carbonyl content of crude microsomes from control and 4-day stimulated EDL and TA muscles of rabbit

	Carbonyl content (nmol/mg protein)		
	contralateral	stimulated	% of control
0-h group	5.355 ± 0.267	8.201 ± 2.776	153
4-h group	4.285 ± 1.307	5.741 ± 0.817	134

Values are means ± S.D. from triplicate measurements on each sample. Protein was determined according to Bradford [25].

detect bityrosine formation by fluorometry is in agreement with this observation. As judged by malondialdehyde determination, lipid peroxidation does also not seem to occur and, therefore, may be excluded as a possible cause of Ca^{2+}-ATPase inactivation under in vivo conditions. This observation agrees with results from in vitro studies in which lipid peroxidation did not seem to be critical to Ca^{2+}-ATPase inactivation [16]. Also, sulfhydryl group oxidation may be excluded as being causally related to the inactivation of Ca^{2+}-ATPase under in vivo conditions, although Ca^{2+}-ATPase inactivation and sulfhydryl group oxidation have been induced in vitro by free radical-generating reactions [41]. However, due to non-physiological conditions and concentrations, the effects of exogenously generated free radicals on isolated membranes may not necessarily be the same as observed in vivo. Moreover, some of the changes induced in vivo might be reversible, e.g. sulfhydryl oxidation. This could explain the failure to detect significant changes in the sulfhydryl group content of microsomes from stimulated muscle.

Our findings clearly point to SR protein oxidation as a major change induced by CLFS and, therefore, the partial inactivation of the Ca^{2+}-ATPase could result from protein oxidation by the action of reactive oxygen species. Indeed, we show an elevated content of DNPH-reactive carbonyls in microsomes from stimulated muscles. In addition, protein modification by tyrosine nitration through endogenously generated peroxynitrite seems to play an important role. SR Ca^{2+}-ATPase was previously shown to be inactivated in vitro by peroxynitrite [41]. This strong oxidant obviously acts the same way in vivo. Thus, as judged from immunochemical detection of nitrotyrosine, the 105 kDa band corresponding to Ca^{2+}-ATPase is strongly labeled in the microsomes from stimulated muscle. The formation of peroxynitrite from NO and superoxide during CLFS is not unexpected. NO synthase is abundant in fast-twitch fibers, especially its ncNOS isoform [22]. Also, the generation of reactive oxygen species in contracting muscle is well established [6]. The production of reactive oxygen species is possibly enhanced by CLFS in the poorly capillarized muscles under study [42] in which sustained contractile activity may lead to functional ischemia. Moreover, the stimulation protocol used, alternating periods of contractile activity and recovery, most likely creates alternating periods of ischemia and reperfusion.

Acknowledgements: This study was supported by the Deutsche Forschungsgemeinschaft, Pe 62/19-3. The authors thank Mrs. Bärbel Gohlsch and Mrs. Elmi Leisner for excellent technical assistance.

References

[1] Hicks, A., Ohlendieck, K., Göpel, S.O. and Pette, D. (1997) Am. J. Physiol. 273, C297–C305.
[2] Heilmann, C. and Pette, D. (1979) Eur. J. Biochem. 93, 437–446.
[3] Leberer, E., Härtner, K.-T. and Pette, D. (1987) Eur. J. Biochem. 162, 555–561.
[4] Dux, L., Green, H.J. and Pette, D. (1990) Eur. J. Biochem. 192, 95–100.
[5] Matsushita, S. and Pette, D. (1992) Biochem. J. 285, 303–309.
[6] Davies, K.J.A., Quintanilha, A.T., Brooks, G.A. and Packer, L. (1982) Biochem. Biophys. Res. Commun. 107, 1198–1205.
[7] Arkhipenko, Y.V., Kagan, V.E. and Kozlov, Y.P. (1983) Biokhimiya 48, 376–383.
[8] Scherer, N.M. and Deamer, D.W. (1986) Arch. Biochem. Biophys. 246, 589–601.
[9] Kukreja, R., Okabe, E., Schrier, G.M. and Hess, M.L. (1988) Arch. Biochem. Biophys. 261, 447–457.
[10] Ohta, A., Mohri, T. and Ohyashiki, T. (1989) Biochim. Biophys. Acta 984, 151–157.
[11] Lee, C.I. and Okabe, E. (1995) Jpn. J. Pharmacol. 67, 21–28.
[12] Ishibashi, T., Lee, C.-I. and Okabe, E. (1996) J. Pharmacol. Exp. Ther. 277, 350–358.
[13] O'Neill, C.A., Stebbins, C.L., Bonigut, S., Halliwell, B. and Longhurst, J.C. (1996) J. Appl. Physiol. 81, 1197–1206.
[14] Hasegawa, A., Suzuki, S., Matsumoto, Y. and Okubo, T. (1997) Free Radical Biol. Med. 22, 349–354.
[15] Xu, K.Y., Zweier, J.L. and Becker, L.C. (1997) Circ. Res. 80, 76–81.
[16] Viner, R.I., Krainev, A.G., Williams, T.D., Schöneich, C. and Bigelow, D.J. (1997) Biochemistry 36, 7706–7716.
[17] Green, H.J., Düsterhöft, S., Dux, L. and Pette, D. (1992) Pflügers Arch. Eur. J. Physiol. 420, 359–366.
[18] Fitts, R.H. (1994) Physiol. Rev. 74, 49–94.
[19] Williams, J.H. and Klug, G.A. (1995) Muscle Nerve 18, 421–434.
[20] Reid, M.B. (1996) News Physiol. Sci. 11, 114–119.
[21] Balon, T.W. and Nadler, J.L. (1994) J. Appl. Physiol. 77, 2519–2521.
[22] Kobzik, L., Reid, M.B., Bredt, D.S. and Stamler, J.S. (1994) Nature 372, 546–548.
[23] Beckmann, J.S., Ye, Y.Z., Anderson, P.G., Chen, J., Accavitti, M.A., Tarpey, M.M. and White, C.R. (1994) Biol. Chem. Hoppe-Seyler 375, 81–88.
[24] Ischiropoulos, H. and Al-Mehdi, A.B. (1995) FEBS Lett. 364, 279–282.
[25] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[26] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[27] Heilmann, C., Brdiczka, D., Nickel, E. and Pette, D. (1977) Eur. J. Biochem. 81, 211–222.
[28] Seidler, N.W., Jona, I., Vegh, M. and Martonosi, A. (1989) J. Biol. Chem. 264, 17816–17823.
[29] Ohkawa, H., Ohishi, N. and Yagi, K. (1979) Anal. Biochem. 95, 351–358.
[30] Andersen, J.P. and Möller, J.V. (1977) Biochim. Biophys. Acta 485, 188–202.
[31] Matsushita, S., Dux, L. and Pette, D. (1991) FEBS Lett. 294, 203–206.
[32] Murphy, A.J. (1976) Biochemistry 15, 4492–4496.
[33] Giulivi, C. and Davies, K.J.A. (1994) Methods Enzymol. 231, 363–371.
[34] Levine, R.L., Williams, J.A., Stadtman, E.R. and Shacter, E. (1994) Methods Enzymol. 233, 346–357.
[35] Guptasarma, P., Balasubramaniam, D., Matsugo, S. and Saito, I. (1992) Biochemistry 31, 4296–4303.
[36] Fountoulakis, M., Juranville, J.-F. and Manneberg, M. (1992) J. Biochem. Biophys. Methods 24, 265.
[37] Meissner, G., Conner, G.E. and Fleischer, S. (1973) Biochim. Biophys. Acta 298, 246–269.
[38] Simoneau, J.-A., Kaufmann, M., Härtner, K.-T. and Pette, D. (1989) Pflügers Arch. Eur. J. Physiol. 414, 629–633.
[39] Leberer, E., Härtner, K.-T., Brandl, C.J., Fujii, J., Tada, M., MacLennan, D.H. and Pette, D. (1989) Eur. J. Biochem. 185, 51–54.
[40] Briggs, F.N., Lee, K.F., Feher, J.J., Wechsler, A.S., Ohlendieck, K. and Campbell, K. (1990) FEBS Lett. 259, 269–272.
[41] Viner, R.I., Hühmer, A.F.R., Bigelow, D.J. and Schöneich, C. (1996) Free Radical Res. 24, 243–259.
[42] Škorjanc, D., Jaschinski, F., Heine, G. and Pette, D. (1998) Am. J. Physiol. (in press).