Myopathy in Broiler Chickens: A Role for Ca\textsuperscript{2+}-Activated Phospholipase A\textsubscript{2}?

D. A. Sandercock\textsuperscript{1} and M. A. Mitchell

Roslin Institute (Edinburgh), Roslin, Midlothian EH25 9PS, United Kingdom

ABSTRACT The role of Ca\textsuperscript{2+}-dependent phospholipase A\textsubscript{2} (PLA\textsubscript{2}) in the mechanism of skeletal muscle damage in broiler chickens was examined in vitro using a novel, synthetic, PLA\textsubscript{2}-specific inhibitor Ro31-499/001 (Ro31). Muscle damage was assessed by measurement of creatine kinase (CK) efflux from isolated muscles into the incubation medium. Treatment with the specific Ca\textsuperscript{2+} ionophore 4-Br-A23187 (5 \(\mu\text{M}\)) caused a 72\% elevation \((P < 0.05)\) in muscle 45Ca\textsuperscript{2+} accumulation, which was associated with a marked increase \((P < 0.001)\) in muscle CK efflux (7.6-fold). Incubation with Ro31 (50 \(\mu\text{M}\)) reduced \((P < 0.001)\) CK efflux from muscles treated with ionophore (45\%) but was without effect on 45Ca accumulation. Treatment with the Na\textsuperscript{+} ionophore monensin (100 \(\mu\text{M}\)) induced 55\% \((P < 0.05)\) elevation in 45Ca\textsuperscript{2+} accumulation with a concomitant 2.5-fold increase \((P < 0.001)\) in CK loss. Muscles incubated with monensin in the presence of Ro31 exhibited a 49\% reduction \((P < 0.001)\) in CK leakage but showed no change in 45Ca\textsuperscript{2+} uptake. The results indicate that increasing external Ca\textsuperscript{2+} entry, directly or indirectly, and elevation of intracellular Ca\textsuperscript{2+}, significantly alters sarcolemmal integrity resulting in increased CK efflux from broiler skeletal muscle. This process is, at least in part, dependent upon activation of PLA\textsubscript{2} activity and thus inhibitable by Ro31. It is further proposed that muscle damage in poultry induced by a range of stresses, and insults may also be mediated by a Ro31 sensitive, PLA\textsubscript{2}-dependent component. The findings have implications for strategies to reduce or prevent myopathies in poultry affecting bird welfare and product quality.

(Key words: broiler, calcium ions, monensin, muscle damage, phospholipase A\textsubscript{2})

INTRODUCTION

Calcium ions (Ca\textsuperscript{2+}) play a major role in skeletal muscle function. Although physiological increases in myoplasmic free Ca\textsuperscript{2+} have a broad range of regulatory actions on numerous cellular processes (e.g., excitation-contraction coupling), high uncontrolled and sustained elevations in intracellular Ca\textsuperscript{2+} cause muscle damage characteristic to many muscle diseases and pathologies (Bodenstein and Engel, 1978; Duncan, 1978). It is now well recognized that prolonged and unchecked increases in myoplasmic Ca\textsuperscript{2+} can activate several intracellular degenerative processes, which result in the structural and functional breakdown of skeletal muscle cells (Jones et al., 1984; Duncan and Jackson, 1987; Jackson, 1993).

Previous in vitro work in our laboratory (Mitchell et al., 1994) has demonstrated that pathological elevations in myoplasmic Ca\textsuperscript{2+} in chicken skeletal muscle induced by a specific Ca\textsuperscript{2+}-ionophore (4-bromo-A23187) cause a marked increase in the loss of the intracellular skeletal muscle isoenzyme creatine kinase (CK; EC 2.7.3.2). Measurement of increases in the plasma or serum activities of this isoenzyme is well established as a reliable diagnostic indicator of skeletal muscle damage (Hamburg et al., 1992). Marked elevations in plasma CK activity have been reported in several pathological muscle conditions in poultry including growth associated and stress induced myopathies (Mitchell et al., 1992; Mitchell and Sandercock, 1994, 1995), antibiotic ionophore (monensin) toxicosis (Horowitz et al., 1988; Sandercock and Mitchell, 1996), and halothane anesthesia (Mitchell et al., 1999). The leakage of CK enzyme from skeletal muscle cells in these situations is believed to be a consequence of alterations in muscle cell membrane (sarcollemmma) integrity induced by elevations in myoplasmic Ca\textsuperscript{2+} (Sandercock and Mitchell, 1998; Sandercock et al., 2001).

There a number of mechanisms through which elevated intracellular Ca\textsuperscript{2+} may induce metabolic dysfunction and cellular damage including altered membrane integrity (Mitchell, 1999). It may be hypothesized that one such mediator of sarcolemmal alteration may involve the activation of the Ca\textsuperscript{2+}-dependent membrane associated enzyme phospholipase A\textsubscript{2} (PLA\textsubscript{2}), EC 3.1.1.4 (Jackson, 1993; Jackson et al., 1984). There is a close correlation between prostaglandin release, resulting from Ca\textsuperscript{2+} stimulation of PLA\textsubscript{2}, and CK efflux from isolated mammalian muscle.
(McArdle et al., 1991). Activation of PLA2 enzyme by micromolar elevations in intracellular Ca2+ (Dennis, 1994; Murakami et al., 1997) results in membrane lipid hydrolysis and generation of pro-inflammatory intermediates such as prostaglandins, thromboxanes, and leukotrienes and membrane-damaging lysophospholipids (Kramer and Sharp, 1997). Physiological activation of PLA2 occurs in skeletal muscle in response to stretch (Vandenburgh et al., 1993). This process also results in stimulation of phospholipid hydrolysis and prostaglandin synthesis. In addition nonspecific inhibition (chlorpromazine and mepacrine) of phospholipase activity results in reductions in protein loss from metabolically inhibited, isolated, mouse skeletal muscle (Jackson et al., 1984).

Recent advances in therapeutic strategies for the treatment of inflammatory and degenerative diseases has led to the development of novel, more specific, synthetic PLA2 inhibitors including Ro31-4493/001 (Ro31) (Dijkstra et al., 1994) to the development of novel, more specific, synthetic PLA2 inhibitors including Ro31-4493/001 (Ro31) (Dijkstra et al., 1994). Reductions in protein loss from perfused isolated guinea pig hearts treated with Ro31, resulting from experimentally induced increases in intracellular Ca2+, have been reported by Sulieman et al. (1994).

The aim of the present study, therefore, was to investigate the role of Ca2+-activated phospholipase A2 in the mechanism of muscle damage and CK loss using the novel PLA2 inhibitor Ro31, employing a previously described isolated, incubated chicken skeletal muscle preparation (Mitchell et al., 1994). Increases in muscle Ca2+ content were induced using a halogenated derivative of A23187 Ca2+ ionophore (4-Br-A23187), which has been reported to exhibit 10-fold greater transport selectivity for Ca2+ over A23187 (Debono et al., 1981). In addition, muscles were incubated with monensin, a Na+ selective polyether-carboxylic ionophore, which is widely employed in the in the poultry industry as an anticoccidial agent (Shumard and Callender, 1968). Muscle damage in poultry associated with monensin overdose has been frequently reported (Chalmers, 1981; Horowitz et al., 1988; Dowling, 1992; Sandercock and Mitchell, 1996). Previous in vitro studies in chicken muscle (Sandercock and Mitchell, 1996) have also demonstrated a concentration dependent increase in myoplasmic Ca2+ accumulation and CK loss with monensin treatment. The work presented herein has previously been reported in part in this journal in abstract form (Sandercock and Mitchell, 1999).

MATERIALS AND METHODS

Birds and Husbandry

The birds used in the study were female broiler chicks obtained from a commercial breeder company. Birds were reared from 1 d old to 42 d of age in pens (3.6 m2) on wood shavings litter. Room temperatures of 18 to 20°C were maintained by controlled ventilation and heating. Birds were provided with ad libitum access to a commercial broiler starter diet and water and were maintained on 14L:10D.

Muscle Isolation and Incubation

At 42 d of age, birds were killed by intravenous injection of sodium pentobarbitone (100 mg/kg body weight). The bilateral tensor patagialis ‘wing web’ muscles (mean weight = 0.110 g) were carefully excised and immediately placed in ice-cold 0.9% saline. The muscles were weighed and mounted (held at resting length) in an isolated tissue incubation chamber containing 2.5 mL complete incubation medium 199 with Earle’s salts. Muscles were incubated at 42°C in pre-equilibrated incubation medium, which was continually gassed with 95% O2 + 5% CO2 at pH 7.4. Muscles were incubated according to a standard incubation procedure developed (Mitchell et al., 1994) from that previously used by Jones et al. (1983) for isolated mouse muscles. The muscles were incubated 150 min, during which the incubation medium was changed at 30-min intervals (5 × 30 min) and replaced with prewarmed fresh medium. An aliquot of used incubation medium (1 mL) was collected and immediately frozen at 20°C for Ca2+ determination. The paired muscles from each bird were randomized between treatments and controls. Four muscles were incubated simultaneously in each experimental run. Eight muscles were used for each treatment or treatment combination. Previous studies (e.g., Mitchell et al., 1994) have demonstrated the viability of the isolated muscle preparation in vitro.

In order to measure muscle Ca2+ accumulation in response to the ionophore treatments, muscles were incubated with radio-isotopic 45CaCl2 (6.4 kBq/ml). Calcium ionophore 4-Br-A23187, Na+ ionophore monensin, and Ro31 were solubilised in 70% ethanol prior to addition to the incubation medium. The PLA2 inhibitor (Ro31) was kindly donated by Geoff Lawton (Roche Research Centre, Welwyn Garden City, Herts, UK). Equivalent volumes of 70% ethanol were added to control muscle incubations.

Preparation of 45Ca2+ Incorporated Muscle Homogenates

At the end of each experimental run muscles were removed from the incubation medium, blotted dry, rapidly frozen in liquid nitrogen, and stored at −20°C pending analysis. The frozen muscles were transferred into tubes containing 4 mL 0.9% ice-cold saline and homogenized for 3 min at 1,200 rpm on ice. 45Ca2+ activity of the muscle homogenate was measured as radioactive disintegrations per minute (dpm) using an automated scintillation counter. Muscle 45Ca uptakes are expressed in disintegrations per minute per gram per wet weight muscle.
CK Measurement

The CK efflux activities were determined in the used incubation media by automated spectrophotometry8 using a commercially available diagnostic kit9 modified for use with avian plasma (Mitchell et al., 1992). Possible spectrophotometric and kinetic interferences by pharmacological treatments on CK activity were ruled out in previous preliminary experiments (Brazeau and Fung, 1989). The CK activities are presented as rates of enzyme efflux per unit time (mU/g per min) and as total enzyme loss per experimental run (U/g per wet weight muscle) from integration of values obtained at every time point.

Statistical Analysis

All data are presented as means ± one standard deviation. Standard ANOVA methods were used to assess the statistical effects of the pharmacological treatments (SAS Institute, 1996). Effects of treatment on rates of enzyme efflux over time were assessed by two-way ANOVA. Treatment effects on integrated measures of total CK loss and muscle45Ca2+ uptake were assessed by one-way ANOVA. Comparisons of differences between two sample means were examined using two-tailed unpaired Student’s t-tests.

RESULTS

Effect of Ro31 on Ca2+ Ionophore-Induced Muscle Damage

Treatment with the Ca2+ ionophore 4 Br-A23187 (5 µM) induced a 12-fold increase (P < 0.001) in the rate of CK efflux from the incubated muscles (Figure 1a), a rate of enzyme loss that remained more or less constant for the duration of the experimental run. Treatment with Ro31 (50 µM) significantly lowered the rate of ionophore-induced CK release (26%; P < 0.05), which continued to fall (41%; P < 0.05) until the end of incubation. Treatment with Ro31 alone had no effect on the rate of CK efflux from muscles incubated in the absence of Ca2+ ionophore and was comparable with rates of enzyme efflux under control incubation conditions (Figure 1b). The effects of Ro31 on muscle45Ca2+ uptake and total integrated CK loss are shown in Figure 2. Treatment with Ro31 had no effect on either muscle45Ca2+ uptake or total CK loss from muscles incubated in the absence of Ca2+ ionophore. Muscles treated with 4-Br-A23187 exhibited a 72% (P < 0.01) increase in 45Ca accumulation compared to controls, which was associated with a 7.6-fold increase in total CK loss. Incubation with the PLA2 inhibitor had no effect on muscle45Ca uptake in the Ca2+ ionophore-treated muscles but reduced total CK loss by 45% (P < 0.001).

Effect of Ro31 on Monensin-Treated Muscles

The effects of Ro31 on monensin treated muscles are shown in Figure 3. Incubation with monensin (100 µM) induced an average 2.4-fold increase in the rate of muscle CK efflux over the duration of ionophore treatment (Figure 3a). Incubation in the presence of Ro31 (50 µM) reduced the average rate of monensin-induced CK release (51%; P < 0.001). The Ro31 had no effect on the rate of CK efflux from muscles incubated in the absence of monensin and was comparable with rates of enzyme efflux from control muscles (Figure 3b). The effects of Ro31

---

8MR 5000, Dynatech Laboratories, West Sussex, UK.
9Biotrol CK Monoreactif, Alpha Laboratories, Hants, UK.
FIGURE 3. The effect of phospholipase A2 (PLA2) inhibitor Ro31-499/001 (Ro31) (50 µM) on creatine kinase (CK) efflux from muscles incubated with the anticoccidial Na+–selective ionophore monensin (100 µM). Monensin-treated muscles incubated with (■) and without (□) PLA2 inhibitor (panel a) control muscles incubated with (○) and without (△) PLA2 inhibitor (panel b). Values represent means ± 1 SD for eight muscles per treatment.

on muscle 45Ca2+ uptake and total CK loss are shown in Figure 4. Incubation with just Ro31 had no effect on muscle 45Ca2+ uptake or total CK loss from muscles incubated in the absence of monensin. Muscles incubated with monensin induced a 55% (P < 0.001) increase in 45Ca accumulation compared with muscle incubated under control conditions and was associated with a 2.5-fold increase in total CK loss. Incubation with the PLA2 inhibitor Ro31 did not affect muscle 45Ca2+ uptake in the monensin-treated muscles but reduced (P < 0.001) total CK loss by 49%.

DISCUSSION

Previous work has suggested that a key step in the pathogenesis of muscle damage (myopathy) may be an increase in the influx of Ca2+ leading to activation of several Ca2+-dependent degenerative pathways and disruptions to muscle cell integrity (Jones et al., 1983, 1984; Jackson, 1993; Mitchell, 1999). Intracellular disruptions include reductions in muscle contractile and metabolic function and the initiation of processes of cellular breakdown including the loss of muscle intracellular constituents such CK, a reliable indicator of myopathy (Hamburg et al., 1992). It is proposed that increases in the loss of this muscle enzyme reflect alterations in muscle membrane (sarcolemmal) integrity induced by a number of Ca2+-dependent pathways including Ca2+-activated enzymes such as PLA2 (Jackson, 1993; Mitchell, 1999). This hypothesis is supported by the demonstration that in immortalized astrocytes, Ca2+ ionophore treatment initiates a cascade involving elevation of intracellular Ca2+, translocation of cytosolic PLA2 to the cell membrane, increased phospholipid hydrolysis, altered membrane integrity, and efflux of the intracellular marker enzyme lactate dehydrogenase (EC 1.1.1.27) (Xue et al., 1999).

The results of the present study suggest that PLA2 indeed may be involved in the development of altered sarcolemmal integrity in isolated muscle in response to elevated intracellular calcium. The CK effluxes resulting from both Ca2+ and Na+ ionophore treatments were reduced (P < 0.001) by the novel, specific PLA2 inhibitor Ro31 (see Figures 2 and 4). There was no concomitant reduction in calcium uptake induced by the ionophore treatments. It therefore may be concluded that the apparent myoprotective effect of Ro31 is exerted at a point in the degradative process beyond elevation of intracellular Ca2+ concentration.

The effects of the PLA2 inhibitor on intracellular enzyme efflux in this study were consistent with the reductions in muscle protein loss observed in previous in vitro studies examining Ca2+-mediated cardiac muscle injury with Ro31 (Sulieman et al., 1994), and with reductions in dinitrophenol induced enzyme loss (CK and lactate dehydrogenase) in other mammalian in vitro studies using nonspecific inhibitors of phospholipase activity such as mepacrine, dibucaine, and chlorpromazine (Jackson et al., 1984, 1987; Phoenix et al., 1990). It has been acknowledged that nonspecific inhibitors of phospholipase activity may exert their effects through mechanisms other than PLA2 inhibition. Unlike Ro31, which was specifically designed to act on the active binding site of PLA2 (Dijkstra et al., 1981a, b), these other inhibitors may act as membrane stabilizers or inhibitors of membrane lipid peroxidation (Jackson et al., 1984, 1987; Phoenix et al., 1990; Jackson, 1993). It is concluded that the results of the present study suggest that Ca2+-activated PLA2 activity may play an important role in the mechanism of skeletal muscle damage associated with entry of external Ca2+ in the chicken.

It has long been recognized that disturbances in cellular Na+ homeostasis may also play a key role in the development of cell damage or injury through its interaction with other ions such as Ca2+ (Trump and Berezesky, 1984; Trump et al., 1989; Tani, 1990). The findings of the current study suggest that the assumed increases in intracellular
Na⁺ concentration ([Na⁺]), caused by treatment with the Na⁺ ionophore anticoccidial agent monensin, can result in an increase in ⁶⁶Ca⁺⁺ accumulation in muscle cells. This process is associated with a concomitant increase in muscle damage as reflected by a greatly elevated CK loss (Figure 4). The increased muscle CK loss induced by monensin is Ro31 inhibitable. This exactly parallels the effect of PLA₂ inhibition by 4-bromo-A23187 on the enzyme efflux resulting from treatment of isolated skeletal muscle with a specific Ca⁺⁺ ionophore, and it may thus be proposed that the two myopathic processes share a common Ro31-sensitive, PLA₂-dependent component.

On the basis of the results presented in this study it may be suggested that Ro31 reduces the in vitro efflux of CK in chicken skeletal muscle via the inhibition of PLA₂ activity downstream of an increase in muscle [Ca⁺⁺]. The latter may result from increased entry of extracellular Ca⁺⁺ as a direct response to the ionophore treatment or may be a consequence of redistribution of intracellular stores of Ca⁺⁺. For example it has been proposed that ionophore-mediated entry of extracellular Ca⁺⁺ may trigger a further release of Ca⁺⁺ from intracellular stores such as the sarcoplasmic reticulum (SR) possibly through a ionophore-mediated entry of extracellular Ca⁺⁺ may trig-

**ACKNOWLEDGMENTS**

The authors thank Geoff Lawton at the Roche Research Centre, Welwyn Garden City, for his kind donation of the PLA₂ inhibitor Ro31-4493/001. Financial support from the UK Department of Environment, Food and Rural Af-

**REFERENCES**


