Praca oryginalna *Original paper*

Ca(2+)-ATPase activity according to the sex and age of Polbar hens

MAGDALENA GRYZIŃSKA, MAGDALENA KRAUZE*, ANETA STRACHECKA

Department of Biological Basis of Animal Production, Faculty of Animals Biology and Breeding, University of Life Sciences, ul. Akademicka 13, 20-950 Lublin
*Department of Biochemistry and Toxicology, Faculty of Animals Biology and Breeding, University of Life Sciences, ul. Akademicka 13, 20-950 Lublin

Gryzińska M., Krauze M., Strachecka A.

Ca(2+)-ATPase activity according to the sex and age of Polbar hens

Summary

The aim of this study was to analyse the physiological values of Ca+2-ATPase catalytic activity in the muscles of 8-, 12- and 18-week-old Polbar breed chickens of both sexes. The material for the analysis consisted of actomyosin homogenate sampled from the greater pectoral muscles (Musculus pectoralis maior) of 20 Polbar hens and 20 Polbar cocks, divided into three groups of 8-, 12- and 18-week-old birds. The studies proved the dependence of the calcium ATPase activity level on the sex and age of the birds examined. Among the three age groups the highest ATPase activity was observed in the birds in their 8th and 12th weeks of life. In the case of the cocks the highest level of the activity was determined in the 8th week of age, with a mean value of 0.37 U/mg, and in the case of the hens in the 12th week, when the mean value amounted to 0.39 U/mg. These results for the ATPase activity in a genetically consolidated hen breed can be used for comparisons with other poultry breeds. The preliminary studies to establish reference values are useful for diagnostic purposes.

Keywords: Ca(2+)-ATPase, Polbar breed, skeletal muscle

Ca^{2+}-ATPase, which belongs to the P-type ATPases present in plasmatic membranes and the endoplasmatic reticulum, is responsible for the removal of an excessive amount of calcium ions from a cell. An increase in the Ca^{2+} ion concentration in cytoplasm generates a reaction to an extracellular stimulus, and a decrease corresponds to the resting phase of the cell. Changes in the calcium ion concentration in cell cytoplasm occur very quickly and may be caused by the ions accumulated inside the cell, as well as in the extracellular fluid. When the cell is in its resting phase, there is an enormous difference between the ion concentration potentials inside and outside the cell, with a resultant high Ca^{2+} ion concentration gradient. The gradient is maintained owing to low membrane permeability and the factors that remove calcium ions from inside the cell. Such factors include the calcium pump drawing energy from ATP and the ion exchanger which replaces sodium ions with calcium ions (1). The obtained results for the ATPase activity in a genetically consolidated hen breed can be used for comparisons with other poultry breeds. The preliminary studies to establish reference values are useful for diagnostic purposes.

The aim of this study was to analyse the physiological values of Ca^{2+}-ATPase catalytic activity in the muscles of 8-, 12- and 18-week-old Polbar breed chickens of both sexes.

Material and methods

The material for the analysis consisted of actomyosin homogenate sampled from the greater pectoral muscles (*Musculus pectoralis maior*) of 20 Polbar hens and 20 Polbar cocks, divided into three groups of 8-, 12- and 18--week-old birds, bred at the Laura Kauffman Didactic and Research Station for Small Animals, which belongs to the Department of Biological Basis of Animal Production at the University of Life Sciences in Lublin. It is a multi--purpose domestic breed developed in the mid-20th century by cross-breeding Greenleg Partridge hens with a Plymouth Rock cock, mixed with the Sussex breed. The effect of the cross-breeding was an autosexing breed, i.e. one in which it is possible to identify the sex of the chicks immediately after they are hatched by their plumage colour. An actomyosin solution was obtained by adding 50 ml of 0.6 M KCL to 2.5 g of the greater pectoral muscle. Next, the ingredients were homogenized for 3 minutes at a speed of $10^4/min^{-1}$.

Determination of the Ca^{2+}-ATPase activity. The enzyme activity is expressed in nanomols of orthophosphate released during one minute from one milligram of protein. The released phosphorus is assayed colorimetrically (3). For each sample the ATPase activity was tested in the

following way: 1000 µl of the actomyosin solution was introduced into two test tubes marked as B – the tested sample – and K – the control sample. As the next step, 200 µl of a 0.01 M $CaCl_2$ solution and 600 µl of a 0.6 M KCl solution were added. One of the substrates, 200 µl of a 0.01 M ATP solution, was poured into the B test tube, while the substrate was poured into the K test tube after thermal inactivation of the enzyme. Both test tubes (B and K) were incubated for 15 minutes in a bath at 37°C. The reaction was interrupted by adding 2000 µl of a mixture consisting of 1.5 M H_2SO_4 and 2.5% ammonium molybdate solutions in the proportion of 3 : 1. The precipitated sediment was filtered. The clear filtrates were spiked with 500 µl of a 10% ascorbic acid solution. After 30 minutes the absorbance of the tested sample was read in relation to that of the control sample at a wave length of 750 nm. The enzyme activity was calculated according to the following formula: Activity (U/mg) = A of the sample × 100/C of the protein (mg/ml) × incubation time. The resultant quantitative data were processed statistically with a software suite containing an SAS test package.

Results and discussion

The enzymatic activity level of the analysed skeletal muscle enzyme of the Polbar hens is presented in Table 1. In the studies conducted, the dependence of the calcium ATPase activity level on the sex and age of the birds was determined. The highest ATPase activity was observed in the birds in their 8th and 12th weeks of life. In particular: in the case of the cocks the highest activity levels were determined in the 8th week of life, with a mean value of 0.37 U/mg, while in the hens the activity levels were the highest in the 12th week, when their mean value was 0.39 U/mg. Considering the sex of the birds as a potential determinant of the enzyme catalytic activity, it is easy to notice differences between the values for hens and cocks, though the differences are not statistically significant. Nevertheless, the greatest contrast in the biochemical index was observable in the 8-week-old hens and cocks.

We found that only in the oldest birds (18 week olds), the ATPase activity was almost identical in males and females, as it amounted to a mean value of 0.28 U/mg. These values represent the lowest ATPase activity index in the age groups analysed and differ considerably from the high values in the 12-week group. The enzyme values for the 18-week-old hens and cocks, i.e. the lowest, also plainly differ from the values for the youngest (8-week-old) birds in the analysed group, especially in the case of cocks. For cocks the activity value at 8 weeks of age was considerably higher than at 18 weeks (and the same as at 12 weeks).

The data pattern obtained from the age-related analyses of the Ca^{2+}-ATPase activity reveals that at 12 weeks all the birds showed the highest enzyme activity. In both sexes it amounted to a mean value of 0.37 U/mg. However, a large decrease in the ATPase activity was observed at 18 weeks of age in both males and females.

The greatest difference in the biocatalyst enzyme levels between the sexes was observed in the youngest, 8-week-old chicks. The enzyme activity levels at different life stages of cocks and hens were not proportional, falling and rising in cocks at dif-ferent times than in hens.

Unfortunately, the available literature provides scarce information about advances in the above area, related to the question of intracellular enzyme activity in Polbar hens of different ages and both sexes. This makes it difficult to objectively evaluate the research conducted or to take a critical stance in relation to it, which also prevents an accurate comparative analysis of the activity values of the given enzyme.

The research proves that the activity of the Ca^{2+}--ATPase located in the muscle SR depends on the age of the animal. As the animals grow, this value increases or remains at a constant level (at the 8th and 12th weeks of age) with a slight decrease in the last of the life stages analysed (18th week). Interesting results were obtained by Lowe et al. (9), who performed studies on rats classified into two age groups of 8-12 month olds and 32-37 month olds. The myosin fibres taken from the older animals displayed a 20% lower contraction force than those from the younger animals. Despite the changes in contraction force, the maximal myosin ATPase activity did not differ between the two groups. Moreover, the muscular force and enzymatic activity at the maximal calcium concentration were positively correlated in the group of the younger animals. This means that the force decreases with age, while ATPase remains at the same level all the time. The maximal ATPase activity amounted to 686 µM/s in the

Tab. 1. ATPase (Ca^{2+}-ATPase) activity in the pectoral muscles of the Polbar breed, depending on their sex and age (n = 20, $\bar{x} \pm s$)

Age (in weeks)	Ca^{2+}ATPase activity (U/mg)		
	♂	♀	♂ + ♀
8	$0.37^{a} \pm 0.12$	$0.24^{b} \pm 0.14$	0.30 ± 0.15
Range	(0.2392 up to 0.6918)	(0.0915 up to 0.5508)	(0.0915 up to 0.6918)
12	$0.34^{ab} \pm 0.11$	$0.39^{a} \pm 0.17$	0.37 ± 0.14
Range	(0.1689 up to 0.5886)	(0.2157 up to 0.6863)	(0.1689 up to 0.6863)
18	$0.28^{b} \pm 0.11$	$0.28^{b} \pm 0.11$	0.28 ± 0.11
Range	(0.1846 up to 0.5250)	(0.1840 up to 0.5050)	(0.1840 up to 0.5250)
Total	0.33 ± 0.12	0.30 ± 0.15	0.31 ± 0.14
Range	(0.1689 up to 0.6918)	(0.0915 up to 0.6863)	(0.0915 up to 0.6918)

Explanations: a, b – the mean values marked with different letters in the columns significantly differed at $P \leq 0.05$

young and 697 μM/s in the old animals, thus showing practically no difference. However, the maximal muscular power, amounting to 162 kN/m^2 in the young, was much lower in the older animals, at only 136 kN/m^2. These studies showed a 10%-40% decrease in the contraction force of muscles taken from rodents. Despite the age-related power deficiency, it was unequivocally proved that the ATPase activity in muscles did not change with age (9).

Such conclusions may appear unexpected and controversial in the light of the publications which suggest age dependence even in the case of the above studies. However, they have their justification. Studies that determine enzyme activities are not common, mainly because of the drastic methods of sampling the material for analysis, which involve killing the animals or, in the case of humans, biopsy and operational sampling of tissues. Therefore, such large divergences in the assessment of research results may be caused by purely technical aspects. There was a diametrical difference in the animals used for the two studies. In the first case they were birds of the domestic Polbar breed, whereas in the second case they were purely laboratory rodents (the Fisher 344 × Brown Norway F1 hybrids). The other researchers used the limb muscle, the so-called semimebraneous muscle, whereas in the present study the pectoral muscle was used.

Lowe et al. (9) found that the contradictions in the scientific conclusions resulted from the temperature of the reactions performed. Lowe's entire experiment was conducted at 21°C, while the calcium ATPase taken from the hen pectoral muscle was analysed at 37°C. The enzymatic activity was examined in different age groups. The rats were investigated at a mature (12 months) and senile age (37 months); the material for analysis in the study on hens was sampled before their puberty.

In 2001, scientists from Pennsylvania isolated actomyosin preparations from three different species – the C57 B1/6 mice, the Wistar rats and humans – dividing each into three age groups. Independently of the species, there was a considerable decrease of 18-25% in the activity and speed of muscular contractions in the isolated preparations. Only in the young and mature individuals it remained at a steady level. The older the animals were, the greater a decrease was observed. In the rats the muscular contraction speed was on average 1.34 μm/s, ranging from 0.89 to 2.19 μm/s, in the young animals, whereas in the old it was significantly lower at an average of 1.00 μm/s and within the range of 0.75 ÷ 1.45 μm/s. In humans these values were analysed depending on the muscular fibre type. The speed of the I-type filaments was on average 0.69 μm/s and within the range of 0.45 ÷ 0.95 μm/s in the young individuals, whereas in the older people it was on average 0.56 μm/s, ranging from 0.39 to 0.66 μm/s. The other fibre type, IIA was characterized by a higher speed than the first one, comprised within the range of 2.26 ÷ 3.16 and with a mean value of 2.63 μm/s in the young and by a lower speed of the older muscles: 2.57 μm/s, (2.30 ÷ 2.79) (7). These studies revealed changes in the functioning and activity of skeletal muscles depending on age. However, the authors focused mainly on evaluating the deterioration of muscular force at a senile age. The increase in the calcium ATPase activity and in the muscular contraction force was not analysed during the adolescence of the individuals and during their ontogenesis (7).

Apart from age-related criteria Frontera et al. (5) also considered the sex of the subjects. The women and men were divided into 3 groups: younger men (at 37.3 years of age on average), older men (74.4 years of age) and older women (72.1). The objective of the experiment was to compare the specific *in vivo* muscular force in the lower limbs (skeletal muscles) of men and women. All the patients underwent biopsy. Muscle fibres sampled from the older men displayed a much lower maximal contraction force than the fibres taken from the younger men. A correlation was also identified between the sex and the muscle fibre type: the differences in the contraction force between the two sexes depended on the fibril type, that is to say, they increased with the fibre size. The smaller fibres (the I A type) in the women were stronger than the smaller ones in the men. In contrast, the thicker fibrils (II A) were much stronger in the men than in the women. Recapitulating, in the females there was no difference in the contraction force between the two fibre types, whereas considerable differences were observed in the males (5).

Similar conclusions to those of Frontera et al. (5) were arrived at by Krivickas et al. (8), who performed a similar experiment on women and men in widely different age groups. It was shown that age and sex affect the contraction speed of the muscles whose efficiency dwindles with age (8).

Age-dependent changes in skeletal muscles were also analysed by Gafni and Yuh (6). They isolated the SR from the skeletal muscles of Spraque-Dawley rats of 4-28 months of age. A decrease in the muscular activity related to age was observed in all the SR proteins isolated from muscle tissue. The decrease was linear; with age there was a progressive loss in the size and mass of the muscles. There was no analogy, however, in the research results in the case of the cardiac muscle tissue. No age-related changes were observed, though the research conducted by Ma et al. (10) proved that changes in Ca^{2+}-ATPase expression in the cardiac muscle lead to serious pathologies in the functioning of the heart (10). An analysis was also made of the calcium content (per one milligram of protein in the SR) in all age groups, but no fundamental differences were found between them. Only in the oldest animals the calcium level was slightly lower. A large decrease occurred, however, in the SR protein concentration and in the calcium sequestration capacity,

i.e. in its uptake. Despite these age-related changes in the muscle functioning, it was confirmed once more that the calcium-ion-stimulated ATPase activity did not change with age. It was then a constant, unlike the efficiency of the active transport across the sarcoplasmatic membrane with the participation of Ca^{2+} ions. The efficiency clearly decreased with age, amounting to 0.37 in the 3rd-4th months of life and only 0.15 in the 24th month. Such changes may be caused by deficiencies and modifications in the SR fraction resulting from the aging process. The same scientists inactivated ATPase from the SR membranes in two ways. In the first process, it lost approximately 75% of its activity. It was concluded that the loss was related to the age of the rats, since the SR isolated from the older animals was more susceptible to changes and modifications than its younger equivalent. The other method involved the use of Triton X-100. Under its influence the calcium ATPase lost only 6% of its initial activity, and, importantly, there was no difference between the kinetic inactivation of the young and old ATPase proteins. These results prove that, regardless of age, modifications in the SR and calcium pump stability affect only the SR membrane, not the ATPase proteins (6). According to Ferrington et al. the Ca^{2+}-ATPase isolated from older muscles displays a faster inactivation during thermal processing compared with young tissue (4).

A similar view is expressed by Berchtold et al. (2) in their publication on the role of calcium ions in skeletal muscles. The scientists confirm that the age-related weakening of the inner SR functions and the loss of its volume in cells are in all probability the cause of the decrease in the contraction speed of the fast skeletal muscles. Additionally, the lack of correlation between the stimulation of the SR membrane and the release of Ca^{2+} ions can be a major determinant of muscular weakness and fatigue progressing with age in rats and humans (2).

The effect of the age of animals on the functioning of the calcium pump in the skeletal muscle SR was also explored by Narayanon et al. (11). The study was conducted on two age groups of Fisher 344 rats – 6-8 and 26-28 month olds – from which fragments of the slowly and quickly contracting muscles were taken. The analysis of the results showed an age-related decrease in the maximal muscle speed. However, the Ca^{2+}-ATPase activity in the SR of the muscles under analysis displayed no substantial age-related differences. In a similar experiment, however, Viner et al. identified a significant decrease in the ATPase activity of approx. 26%, but only in the SR isolated from the slowly contracting muscles, not from the fast muscles, in the case of which the activity did not change (13). Age-related changes were also imperceptible in the activity of the RyR receptor, or the calcium releasing channel, and of the Ca^{2+}-binding proteins, such as calmodulin or calsequestrin. The conclusion from those studies was that a weakening of the calcium pump in some muscles took place because of the separation of the hydrolysis and Ca^{2+} transport processes, which made for a slower muscular decontraction in the older animals (11).

In 2005, Sepúlveda et al. (12) published the results of a research on the calcium ATPase activity in a developing brain of White Leghorn hens on the 10th, 12th, 15th and 18th days of their embryogenesis and immediately after being hatched. The enzymatic activity was measured at 37°C by the spectrophotometric method at a wavelength of 340 nm. The objective of the analysis was to determine when and where in the neural tissue the calcium pump underwent expression during embryogenesis and whether its activity varied with age and stage of development. It was proved that both the sarcoplasmatic reticulum (SERCA) Ca^{2+}-ATPase and the plasmatic membrane (PMCA) Ca^{2+}-ATPase started functioning at the earliest point in the embryogenesis. SERCA already appeared on the 12th day of the embryogenesis and amounted to 0.03 μmol·min(–1)·mg(–1); on the 15th day of the embryogenesis it amounted to 0.05 μmol·min(–1)·mg(–1), and immediately after the chicks were hatched the coefficient amounted to 0.09 μmol·min(–1)·mg(–1), showing a growing trend. The RE cisterns were formed after the 12th day of the embryogenesis. This phenomenon was ascribed to the activation of endogenous activators, such as calmodulin or protein kinase, at early developmental stages. It was also confirmed that the activity of both ATPases increased with age and tissue development (12).

All the experiments presented here do not suffice to formulate unambiguous conclusions about the functioning of Ca^{2+}-ATPase in skeletal muscles of animals at the initial stage of their life. The research was mainly aimed at explaining the problem of the weakening of muscular functions with the passage of time. It was a response to the high demand for such studies, which may prove important to the clinical diagnostics of human diseases and conditions.

An in-depth analysis of all the research leads to the conclusion that the Ca^{2+}-ATPase activity does not change despite a decrease in the muscular contraction speed, a decrease in the calcium concentration and changes in the SR. It is unknown, however, how the enzyme behaves during the later development and adolescence of animals. What is known is that the calcium pump parameters are different and specific for each muscle type.

The same reasoning suggests that the Ca^{2+}-ATPase activity does not change as the animal grows older. Yet, such a hypothesis seems erroneous in the light of the above studies, all the more so since the cited publications deal with mature or senile animals, not with the young, which are still developing towards full maturity. The only scientific conclusions that can provide a comparative model are those arrived at on the basis of the embryogenesis of hens.

References

1. *Angielski S., Rogulski J.*: Biochemia kliniczna. PZWL, Warszawa 1991.
2. *Berchtold M. W., Brinkmeier H., Müntener M.*: Calcium ion in skeletal muscle: its crucial role for muscle function, plasticity, and disease. Physiol. Rev. 2000, 80, 1215-1265.
3. *Chen P. S., Toribara T., Warner H.*: Microdetermination of phosphorus. Anal. Chem. 1956, 28, 1756-1758.
4. *Ferrington D. A., Jones T. E., Qin Z., Miller-Schlyer M., Squier T. C., Bigelow D. J.*: Decreased conformational stability of the sarcoplasmic reticulum Ca-ATPase in aged skeletal muscle. Biochim. Biophys. Acta 1997, 1330, 233-247.
5. *Frontera W. R., Suh D., Krivickas L. S., Hughes V. A., Goldstein R., Roubenoff R.*: Skeletal muscle fiber quality in order men and women. Am. J. Physiol. Cell Physiol. 2000, 279, 611-618.
6. *Gafni A., Yuh K. C.*: A comparative study of the Ca^{2+}-Mg^{2+} dependent ATPase from skeletal muscles of young, adult and old rats. Mech. Ageing Dev. 1989, 49, 105-117.
7. *Höök P., Sriramoju V., Larsson L.*: Effect of aging on action sliding speed on myosin from single skeletal muscle cells of mice, rats, and humans. Am. J. Physiol. Cell Physiol. 2001, 280, 782-788.
8. *Krivickas L. S., Suh D., Wilkins J., Hughes V. A., Roubenoff R., Frontera W. R.*: Age- and gender-related differences in maximum shortening velocity of skeletal muscle fibers. Am. J. Phys. Med. Rehabil. 2001, 80, 447-455.
9. *Lowe D. A., Thomas D. D., Thompson L. V.*: Force generation, but not myosin ATPase activity, declines with ages in rat muscle fibers. Am. J. Physiol. Cell Physiol. 2002, 283, 187-192.
10. *Ma H., Sumbilla C. M., Farrance I. K. G., Klein M. G., Inesi G.*: Cell-specific expression of SERCA, the exogenous Ca^{2+} transport ATPase, in cardiac miocytes. Am. J. Physiol. Cell Physiol. 2004, 286, 556-564.
11. *Narayanon N., Jones D. L., Xu A., Yu J. C.*: Effect of aging on sarcoplasmic reticulum function and contraction duration in skeletal muscles of the rat. Am. J. Physiol. 1996, 271, 1032-1040.
12. *Sepúlveda M. R., Hidalgo-Sánchez M., Mata A. M.*: A developmental profile of the levels of calcium pumps in chick cerebellum. J. Neurochem. 2005, 95, 673-683.
13. *Viner I. R., Ferrington A. D., Williams T. D., Bigelow D. J., Schöneich C.*: Protein modification during biological aging: selective tyrosine nitration of the SERCA 2a isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase in skeletal muscle. Biochem. J. 1999, 340, 657-669.

Corresponding author: dr Magdalena Gryzińska, ul. Akademicka 13, 20-950 Lublin; e-mail: magdalena.gryzinska@up.lublin.pl