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Expression and localization of Ca^{2+}-ATPase in the uterus during the reproductive cycle of king quail (*Coturnix chinensis*) and zebra finch (*Poephila guttata*) ☆

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Abstract

Calcium ATPase (Ca^{2+}-ATPase) is a key enzyme that participates in the translocation of calcium in the uterus of oviparous amniotes during eggshell formation. We used Western blot and indirect immunofluorescence microscopy to determine expression and localisation of uterine Ca^{2+}-ATPase during the reproductive cycle of king quail and zebra finch. The pattern of Ca^{2+}-ATPase expression and localisation during the reproductive cycle was similar for both species. Immunoblots of uterine extracts from quail and finch indicated that Ca^{2+}-ATPase expression is reduced in non-reproductive compared to reproductive females. Similarly, in non-reproductive females, weak apical immunofluorescent staining of Ca^{2+}-ATPase is localised to epithelial cells in a small number of uterine tubular glands. A large increase in apical immunofluorescent staining of tubular gland epithelia occurs in both vitellogenic and reproductive females. The presence of Ca^{2+}-ATPase on the apical surface of tubular gland epithelial cells suggests that the enzyme is involved in the translocation of calcium out of the tubular gland epithelia and into the concentrated fluid of the uterine lumen. Presence of Ca^{2+}-ATPase in vitellogenic females indicates that the enzyme is expressed prior to the time of ovulation and eggshell calcification.

Keywords: Calcium transport; Eggshell; Oviparity; Protein expression; Reproductive cycle

1. Introduction

Calcification of the avian eggshell is characterised by rapid and massive up-regulation in calcium transport from the blood supply to the lumen of the shell gland (Lundholm, 1997; Nys et al., 1999). Eggshell formation typically occurs over 10–20 h post-ovulation (Nys et al., 2004) during which time the rate of calcium deposition may reach as high as 4.4 mg cm^{-2} h^{-1} (Lundholm, 1997). The high rate of calcium translocation is accomplished by means of transporter enzymes whose function is to pump Ca^{2+} against a steep electrochemical gradient to create a concentrated calcium environment in which eggshell mineralisation can occur (El Jack and Lake, 1967; Pike and Alvarado, 1975; Eastin and Spaziani, 1978; Wasserman et. al., 1991).

Calcium-activated adenosine triphosphatase (Ca^{2+}-ATPase), a key enzyme that mediates calcium transport in the uterus of oviparous amniotes (e.g. birds and reptiles) during eggshell formation (Pike and Alvarado, 1975; Wassermann et al., 1991; Lundholm, 1997; Thompson et al., 2007), is a highly conserved ~140 kDa membrane protein (Borke et al., 1989). Ca^{2+}-ATPase activity and expression are associated with periods of eggshell calcification when high concentrations of calcium are required for eggshell formation (Pike and Alvarado, 1975; Coty and Mc Conkey, 1982; Yamamoto et al., 1985; Watanabe et al., 1989; Wassermann et al., 1991). Because eggshell production comprises a relatively brief part of the reproductive cycle, followed by a long

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period of no reproduction, Ca^{2+}-ATPase should be expressed at low levels during non-calcifying periods of the reproductive cycle. Ca^{2+}-ATPase expression throughout the avian reproductive cycle, including the non-reproductive phase, however, has not been characterised.

The purpose of this study was to investigate changes in patterns of expression and localisation of Ca^{2+}-ATPase during reproductive and non-reproductive phases of the avian reproductive cycle. We predicted that high levels of Ca^{2+}-ATPase expression are associated with periods of eggshell calcification whereas low levels of expression are associated with non-reproductive periods. To test these predictions, we characterised changes in uterine Ca^{2+}-ATPase protein expression and localisation during the reproductive cycle of king quail (*Coturnix chinensis*) and zebra finch (*Poephila guttata*). These species were chosen because they represent two major avian phylogenetic lineages (Galliformes and Passeriformes, respectively). Both species exhibit a seasonal reproductive pattern with reproduction beginning in the early spring in response to increasing photoperiod (van Tienhoven, 1968; Sharp, 1996).

2. Materials and methods

2.1. Animal care and tissue collection

Sexually mature king quail (*Coturnix chinensis*) ($n=20$) and zebra finch (*Poephila guttata*) ($n=20$) were obtained from commercial suppliers and maintained in commercial avian enclosures (750 mm × 280 mm × 280 mm, 2 individuals/enclosure) in an animal facility at the University of Sydney. Birds were fed (commercial bird seed supplemented with shell grit and fresh leafy greens) and watered daily.

Birds were initially maintained under a photoperiod of 10:14 h light/dark for one week to ensure that individuals remained in a non-reproductive state. Photoperiod was increased by 1 min every two days for one week, followed by an increase of 2 min every 2 days (max photoperiod = 10.25 h : 13.75 h light/dark) during which time females and males were housed in pairs until reproduction was initiated. Quail and finch hens were assigned to three reproductive stages for sampling; non-reproductive (<4 mm, non-yolky ovarian follicles), vitellogenic (large, >5 mm yolky follicles), and reproductive (eggs in the oviduct or within one day after oviposition). Females were euthanized at the appropriate reproductive stage by cervical dislocation, and the uterus was excised by cutting directly above the vagina and below the isthmus in non-gravid and vitellogenic females and directly above and below the egg in reproductive females. The condition of uterus, position and stage of eggs (newly ovulated, shelled, non-shelled) were noted. After removal, tissues were immediately processed for Western blot or immunofluorescence microscopy (described below).

2.2. Western blots

Ca^{2+}-ATPase expression in uterine tissues was assessed using Western blot analysis. Uterine tissues from non-reproductive (quail $n=2$; finch $n=3$), vitellogenic (quail $n=3$; finch $n=0$), and reproductive (quail $n=2$; finch $n=3$), females were homogenized in Cell Lysis Buffer (Sigma, St Louis MO, USA) and total protein concentration quantified using a Micro BCA assay (Pierce, Rockford IL, USA) according to manufacturers protocol. Because only a single vitellogenic female finch was available at the time of sampling, we did not allocate tissue from this individual for Western blot analyses. Protein concentrations were adjusted to 1 $\mu g\ \mu L^{-1}$ using Laemmli buffer (250 mM Tris, 0.12% bromophenol blue, 40% glycerol, 8% SDS) and heat denatured (40 °C, 30 min). Twenty micrograms of total protein was resolved by 7% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Imobilon-P, Millipore, Billerica, MA, USA). Transfer and equal loading of proteins were confirmed by Ponceau-S staining. Membranes were blocked with 5% nonfat dry milk dissolved in Tris-buffered saline plus 0.05% Tween-20 (TBST) for 1 h at room temperature. Membranes were incubated with mouse monoclonal antibody (Sigma-Aldrich, St Louis MO, USA, No. A7952) recognising Ca^{2+}-ATPase (0.5 $\mu g\ mL^{-1}$ in 5% blocking buffer) overnight at 4 °C. The monoclonal anti-Ca^{2+}-ATPase antibody was predicted to react with quail and finch Ca^{2+}-ATPase protein due to >80% sequence identity between immunogen and amino acid sequence alignments from zebra fish (Genbank accession no. NP00100132), chicken (Genbank accession no.XP418055), mouse (Genbank accession no. NP080758), pig (Genbank accession no. NP999517), and human (Genbank accession no. NP00103822). Membranes were washed 4 times in blocking buffer, followed by 1 wash in TBST, then incubated with horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (Amersham Biosciences, Piscataway, NJ, USA, NA931) 1:5,000 (in TBST) for 2 h at room temperature. Membranes were washed 5 × in TBST then developed with ECL reagent (Millipore Billerica MA, USA). Chemiluminescent bands were visualized using a FluorChem SP imaging system (Alpha Innotech, San Leandro CA, USA).

2.3. Immunofluorescence microscopy

Ca^{2+}-ATPase was localised in uterine tissue from non-reproductive (quail $n=2$; finch $n=9$), vitellogenic (quail $n=6$; finch $n=1$), and reproductive (quail $n=10$; finch $n=10$) females using indirect immunofluorescent microscopy. Freshly excised uterine tissues were laid flat on a piece of wax support, immediately covered in O.C.T. mounting medium (Tissue-Tek,

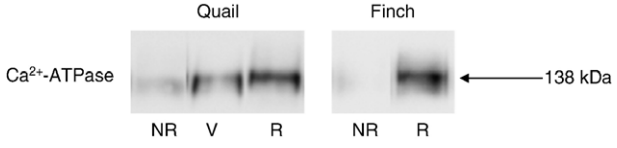

Fig. 1. Western blot of Ca^{2+}-ATPase protein in uterine tissues of king quail (*Coturnix chinensis*) and zebra finch (*Poephila guttata*) at different reproductive stages: non-reproductive (NR), vitellogenic (V), and reproductive (R). Results shown are representative of 2–3 biological (non-reproductive :quail $n=2$; finch $n=3$, vitellogenic: quail $n=3$; finch $n=0$, reproductive :quail $n=2$; finch $n=3$), and experimental ($n=3$) replicates.