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Ex utero culture of viviparous embryos of the lizard, *Zootoca vivipara*, provides insights into calcium homeostasis during development

Tom W. Ecay [a,*], James R. Stewart [b], Greg Wiessner [a], Benoit Heulin [c]

[a] Department of Biomedical Science, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, TN, USA
[b] Department of Biological Sciences, East Tennessee State University, Johnson City, TN 37614, USA
[c] CNRS UMR 6553, Station Biologique de Paimpont, 35380 Paimpont, France

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ABSTRACT

The chorioallantoic membrane resides adjacent to either the inner surface of the egg shell or uterine epithelium in oviparous and viviparous reptiles, respectively. Chorionic cells face the shell or uterine epithelium and transport calcium to underlying embryonic capillaries. Calcium transport activity of the chorioallantois increases in the final stages of development coincident with rapid embryonic growth and skeletal ossification. We excised embryos from viviparous *Zootoca vivipara* females at a stage prior to significant calcium accumulation and incubated them *ex utero* with and without calcium to test the hypothesis that chorioallantois calcium transport activity depends on developmental stage and not calcium availability. We measured calcium uptake by monitoring incubation media calcium content and chorioallantois expression of calbindin-D_{28K}, a marker for transcellular calcium transport. The pattern of calcium flux to the media differed by incubation condition. Eggs in 0 mM calcium exhibited little variation in calcium gain or loss. For eggs in 2 mM calcium, calcium flux to the media was highly variable and was directed inward during the last 3 days of the experiment such that embryos gained calcium. Calbindin-D_{28K} expression increased under both incubation conditions but was significantly higher in embryos incubated with 2 mM calcium. We conclude that embryos respond to calcium availability, yet significant calcium accumulation is developmental stage dependent. These observations suggest the chorioallantois exhibits a degree of functional plasticity that facilitates response to metabolic or environmental fluctuations.

1. Introduction

Most vertebrate embryos accumulate calcium during development along a trajectory that parallels embryonic growth in mass and length. For reptiles and mammals, growth and calcium accumulation accelerate in later embryonic stages. Depending on mode of reproduction, the calcium acquired during development originates from maternally supplied yolk stores and either the calcified egg shell in oviparous reptiles or by placental transfer from uterine secretions in viviparous reptiles and mammals. Mammals are exclusively dependent on placental calcium transfer during prenatal development, whereas crocodilians and birds derive 80% or more of hatchling calcium from the eggshell (Packard, 1994). Species of squamate reptiles have converged on each of these divergent patterns of embryonic calcium nutrition (Stewart and Ecay, 2010). Oviparous squamates exhibit the greatest diversity in the relative contribution of yolk and eggshell calcium to embryonic development of any amniote lineage and, with >1500 viviparous species (Blackburn, 2006), pattern of embryonic calcium nutrition of viviparous species is equally diverse (Stewart and Ecay, 2010). Early speculation that development in squamate species is highly dependent on calcium-enriched yolk and seemingly independent of eggshell calcium (Simkiss, 1967) supported a hypothesis that transitions from oviparity to viviparity are not constrained by the loss of eggshell calcium that accompanies prolonged uterine egg retention culminating in live birth (Packard et al., 1977). However, subsequent descriptions of developmental calcium accumulation in oviparous and viviparous squamate species demonstrate that 1) eggshell calcium can account for up to 80% of hatchling calcium in species with geographic variation in mode of reproduction, i.e., species with both oviparous and viviparous populations (Stewart et al., 2009a), 2) calcium placentotrophy can precede complete loss of eggshell calcium (Linville et al., 2010), and 3) placental calcium transport can compensate fully for loss of eggshell calcium (Ramírez-Pinilla et al., 2011; Stewart and Thompson, 1993). These observations suggest that eggshell thinning with loss of calcium and calcium placentotrophy are not consecutive but coincident evolutionary events and further suggest that mechanisms defending embryonic calcium accumulation during development confer an adaptive advantage to hatchlings and neonates. A consequent hypothesis is that the plasticity of uterine and embryonic calcium transport mechanisms, which can maximize

* Corresponding author.
E-mail address: ecay@etsu.edu (T.W. Ecay).

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calcium accumulation during prolonged uterine egg retention, is an important facilitator of the many transitions from oviparity to viviparity in squamates.

Embryonic accumulation of calcium from the eggshell or uterine secretions is mediated by the chorioallantoic membrane (Packard and Clark, 1996). This tissue, which lies adjacent to the inner surface of the eggshell or the uterine epithelium depending on mode of parity, expresses protein markers for transcellular calcium absorption found in renal and intestinal epithelia (Hoenderop et al., 2005). These include the intracellular calcium binding protein calbindin-D_{28K} and a plasma membrane calcium-transporting ATPase (PMCA). Expression of calbindin-D_{28K} and PMCA in the chorioallantois increases coincident with calcium transport to the embryo in late stages of development suggesting that transcellular calcium movement is an important mechanism for embryonic calcium accumulation (Stewart and Ecay, 2010). Existence of a paracellular calcium transport pathway is also possible but remains to be investigated.

Zootoca vivipara is one of three lizard species that demonstrate geographic variation in reproductive mode (Heulin et al., 1993). As with other oviparous reptiles, the eggshell of oviparous *Z. vivipara* consists of an inner matrix of fibrous protein covered with a well-developed outer layer of calcium carbonate crystals from which the underlying chorioallantoic membrane extracts calcium (Heulin, 1990; Heulin et al., 2002, 2005). Viviparous *Z. vivipara* eggs are surrounded by a thin proteinaceous eggshell positioned between the uterine epithelium and the chorioallantoic membrane (Heulin, 1990; Heulin et al., 2005). Uterine calcium secretions do not accumulate as a precipitated calcium carbonate layer on this eggshell, but pass through to the chorioallantoic membrane (Stewart et al., 2009a). Embryos of each reproductive mode accumulate calcium slowly until late in development, when uptake increases greatly, coincident with increased expression of calbindin-D_{28K} and PMCA in the chorioallantoic membrane (Stewart et al., 2011, Stewart et al., 2009a). The timing of embryonic uptake of calcium may be driven by differences in calcium availability, or alternatively, by the developmental expression of physiological mechanisms for calcium transport. The timing of uterine calcium delivery differs in each reproductive mode as the oviparous uterus calcifies an eggshell early in development and the viviparous uterus provides calcium at later stages coincident with embryonic demand and transport capacity of the chorioallantoic membrane.

We have surgically removed and cultured embryos from viviparous female *Zootoca vivipara* in incubation media with differing calcium content while simultaneously monitoring calcium flux between the embryo and incubation media to test the hypothesis that calcium uptake (chorioallantois calcium transport) is dependent on stage of development and not calcium availability.

2. Methods and materials

2.1. Incubation

Female viviparous *Zootoca vivipara* were collected from Paimpont France in September 2009. Lizards were maintained through a hibernation cycle, males and females paired for mating, and shipped to East Tennessee State University in March 2010. Eggs were removed from females ($N = 6$) prior to the phase of greatest embryonic growth (embryonic stages 36, 37, 37, 37, 37, 38). Embryonic development was staged by the system of (Dufaure and Hubert (1961). Eggs were assigned to one of three treatments, 1) initial sample, 2) incubation in nominally calcium-free media (composition in mM; 137 NaCl, 2.7 KCl, 1 $MgCl_2$, 12 $NaHCO_3$, and 0.3 NaH_2PO_4), or 3) incubation in media + 2.0 mM calcium chloride.

Following the protocols developed by Panigel (1956) and Bleu (2011) eggs were positioned on sterile cotton in a 60 ml plastic culture dish with 20 ml of medium and incubated at 25 °C in a humidified chamber. A media sample (10 ml) was removed for calcium determination and replenished with fresh incubation media (10 ml) at two day intervals until day 11, when the experiment was terminated. Calcium concentration in recovered media was measured with a calcium electrode (MI-600, Microelectrodes Inc., Bedford, NH) attached to an Accumet AB15 pH meter recording in mV mode. Culture dishes with cotton and media but no eggs were used as blanks to control for evaporative concentration of calcium, which was determined to be negligible. The calcium concentration in media samples was measured at the time of collection and electrode calibration curves were obtained at the same time using calcium standards from 100 to 0.01 mM. Calcium flux between compartments (egg and dish) was determined as the difference between the electrode measured calcium and the predicted calcium based on the measured media concentration corrected for carry over from the previous sample period.

2.2. Calcium analysis

Yolks, embryos and eggshells from the initial sample of eggs ($N = 6$ clutches, one egg per clutch per treatment) and from day 11 eggs (embryonic stage 40) ($N = 6$ clutches, one egg per clutch per treatment) were placed in separate tared vials, weighed and frozen (−10 °C). Samples were lyophilized to constant mass in a Labconco Freezone 4.5 freeze dryer. Preparation of samples for calcium analysis followed the method of (Shadrix et al., 1994). Samples were digested in borosilicate glass test tubes containing 3 ml concentrated nitric acid for 3 h at 125 °C. Digestates were cooled to room temperature for 1 h and 1 ml 30% hydrogen peroxide was added. The temperature was returned to 125 °C and maintained for approximately 15 h. The digestates were evaporated gently to near dryness on a hot plate, diluted in hydrochloric acid (1:1 in distilled H_2O) and brought to a final volume of 2.5% hydrochloric acid with distilled water. Lanthanum chloride (1:10) was added to each sample prior to analysis for calcium. Calcium content was estimated using a Shimadzu model AA-6300 atomic absorption spectrophotometer calibrated against samples of known calcium concentration.

2.3. Immunoblotting

Chorioallantoic membranes were isolated from the initial sample of eggs (stage 36–38 embryos) and from eggs after 11 days in culture (stage 40 embryos). Tissue samples were homogenized and fractionated by polyacrylamide gel electrophoresis as described previously (Stewart et al., 2011). Electroblotted proteins were incubated with a rabbit polyclonal antibody to recombinant corn snake calbindin-D_{28K} (Ecay and Stewart, unpublished) at 1:50,000 dilution followed by peroxidase-conjugated anti-rabbit IgG (1:200,000). Immune complexes were visualized on X-ray film by chemiluminescence (Immobilon; Millipore). Blots were stripped and reprobed with anti-actin IgG (1:50,000 dilution; Sigma) as an internal control for sample loading. Films were digitized on a flatbed scanner and quantified by densitometry (Silk Scientific, Orem, UT).

2.4. Statistics

Differences in variance in calcium flux in incubation media between treatments (saline, 2 mM calcium) were tested by a two sample test for variances. We tested for homogeneity of variances within treatments with Bartlett's test. Variation in calcium flux in incubation media with 2 mM calcium was analyzed by one-way analysis of variance and calcium flux in incubation media with saline by both one-way analysis of variance and the Kruskal-Wallis non-parametric test. We analyzed variation in calcium content of egg shells, yolks and embryos with treatment (initial sample, saline, 2 mM calcium) and tissue as fixed factors, tissue dry mass as a covariate and clutch (female) as a random factor. We analyzed variation in calcium content of shell-free eggs (embryos + yolk) among treaments by analysis of variance with treatment as a fixed factor and clutch (female) as a random factor.