The Journal of Experimental Biology 211, 1394-1401
Published by The Company of Biologists 2008
doi:10.1242/jeb.014282

Is there life in the horny layer? Dihydropyridine and ryanodine receptors in the skin of female and male chickens (*Gallus domesticus*)

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Accepted 18 February 2008

SUMMARY

Previous findings in pigeons and chickens show that Ca^{2+} may be accumulated inside the cornified skin cells and that Ca^{2+} microenvironments with a lower- or higher-than-blood concentration may exist in the skin. It has been suggested that the skin may function as a secretory pathway or a reservoir for Ca^{2+} recycling. To test this hypothesis, we studied the dermis and epidermis of female and male chickens *in vivo* to find out whether cellular mechanisms exist for the accumulation, recycling or secretion of Ca^{2+}. For calcium influx and intracellular Ca^{2+} release, respectively, the density of dihydropyridine receptors (DHPRs) and ryanodine receptors (RyRs) was examined, using high-affinity (–)-enantiomers of dihydropyridine and ryanodine labelled with fluorophores. To investigate Ca^{2+} utilization in the skin, the systemic and local activity of the enzyme alkaline phosphatase (ALP) and the concentration of ionic Ca^{2+} were measured in plasma and in cutaneous extracellular fluid, collected by suction blister technique. We found that both DHPRs and RyRs were present in all skin layers from dermis to horny layer. However, receptor densities were highest in the surface layers. With a basic calcium-rich diet, receptor densities were higher in males, particularly in the dermis and mid-epidermis. After a reduction in the nutritional Ca^{2+} input, receptor densities in males decreased to the same level as in females, in which the receptor densities were not affected by the amount of Ca^{2+} in the diet or that resulting from coming out of lay. The extracellular concentration of ionic Ca^{2+} *per se* was not found to affect the density of DHPRs and RyRs in the skin. Spatially, RyRs seem to be located in the periphery of the sebokeratinocyte. ALP activity was shown to be lower in the extracellular fluid than in the plasma in both sexes. However, activity in both extracellular domains increased significantly in females that had come out of lay. This was probably connected with the increased osteoblast activity related to the reformation of structural bone. In conclusion, voltage-sensitive L-type Ca^{2+} channels for ion influx and RyRs for Ca^{2+} release are present in the cells of the skin of female and male chickens. Higher densities in the males receiving excessive Ca^{2+} imply an increased capacity for Ca^{2+} influx and intracellular processing. Even though the functional interactions between DHPRs and RyRs in the sebokeratinocytes could not be demonstrated, peripheral colocation and high receptor densities at the level of exocytosis of the lamellar bodies point to their role as part of a signalling pathway for secretion. The finding that DHPRs and RyRs are present in the horny layer implies that the function of the outermost skin might be more active than had been previously thought and that this function might be both secretory and sensory.

Key words: DHPR, RyR, calcium, skin, avian.

INTRODUCTION

By its primary function, skin is a protective barrier. It provides a first frontier of defence against a wide variety of physical and organic environmental threats and against the loss of body fluids. The protective characteristics of the skin are associated with its anatomical construction, the integrity of its proteinaceous cell envelope, the extracellular lipid barrier (Norlen, 1999; Elias, 2004), and its ability to withhold water in the horny layer (stratum corneum, SC), which seems to be essential for normal epidermal function (Segre, 2003; Rawlings and Harding, 2004). Skin also functions as a secretory organ by excreting fluid and sebum from specialized glands. In birds that are devoid of glands covering the whole body surface, secretion takes place by structural and functional plasticity of the interfollicular epithelium (Menon et al., 1981; Menon et al., 1996; Peltonen et al., 1998; Peltonen et al., 2000).

The skin may also have a role in calcium metabolism. It has been established that the amount of ionic Ca^{2+} is strictly regulated, and that blood, interstitial fluid and tissues, especially bone, form a functional unit that buffers the excessive change in the amount of biologically active Ca^{2+}. In chickens, the major fluctuations in ionic calcium concentration take place in laying females within the daily cycle, the lowest values being displayed at the time of egg calcification (Simkiss, 1967; Luck and Scanes, 1979; Etches, 1996). At the same time, at the site of deposition the local uterine Ca^{2+} values may be increased four- to twelvefold, creating a local high-calcium microenvironment (Arad et al., 1989). In addition to the uterus, skin may also contain calcium microenvironments. It was shown that in a regular 'layer's diet', the amount of ionic Ca^{2+} in the cutaneous interstitial fluid of male chickens started to increase after the completion of growth, while in females the Ca^{2+} remained in equilibrium between these compartments until they came out of lay (Peltonen et al., 2006). As shown in cardiomyocytes, calcium overload may cause enhanced calcium entry (Katra and Laurita, 2007). If this holds for the skin, accumulation of ionic Ca^{2+} may enhance the influx of Ca^{2+} into the skin cells. During cell

differentiation, Ca^{2+} may be either 'entrapped' in the corneocytes and lost *via* desquamation, or associated with some secretory pathway and released into the extracellular space at the transitional interface between the stratum transitivum and corneum (ST-SC). To test this hypothesis, we determined whether transport systems for plasmalemmal Ca^{2+} influx and Ca^{2+} release for possible intracellular signalling exist in the skin of chickens, and whether the densities of the receptors for these vary in relation to sex, the level of the nutritional Ca^{2+} input, the concentration of ionic Ca^{2+} outside skin cells, or the skin layer. Regarding Ca^{2+} influx, we examined the density and distribution of the dihydropyridine receptor (DHPR), the ion-conducting α_1-subunit of L-type calcium channels, by labelling it with a high-affinity enantiomer of DHP. As to the intracellular Ca^{2+} release, we examined the density and distribution of the ryanodine receptor (RyR), also by labelling it with a high-affinity enantiomer of ryanodine. Voltage-gated L-type Ca^{2+} channels typically mediate Ca^{2+} influx in response to depolarization of the plasma membrane. Coupled to or in close proximity with DHPRs, Ca^{2+}-releasing RyRs form an apparatus for Ca^{2+}-induced calcium release that regulates cell functions such as contraction, secretion, neurotransmission and gene expression in many different cell types (Catterall et al., 2005; Coronado et al., 1994).

We also measured the activity of the enzyme alkaline phosphatase (ALP) to indicate Ca^{2+} utilization. ALPs are a group of enzymes that are widely expressed within tissues and catalyse hydrolysis of monophosphate esters at alkaline pH. They are present in soluble and membrane-bound form, the latter anchored by glycosyl phosphatidylinositol to the outer leaflet of the plasma membrane (Moss, 1997). Plasma ALP activity has been associated with bone-forming activity by osteoblasts, and with other tissues that have high cellular turnover (Bell, 1971). Since the tissue-non-specific ALP is synthesized in the skin (Yamashita et al., 1987; Crawford et al., 1995; Hui and Tenenbaum, 1995), and may be involved in physiological and pathological mineralization in tissues other than bone or cartilage (Hui et al., 1997), we also measured its activity in the cutaneous interstitial fluid.

MATERIALS AND METHODS

Ethical considerations

All the experiments conformed to the guidelines for proper animal care and use and had been authorized by the local ethical committee of University of Helsinki, Finland.

Animals

Eight female and male White Leghorn chickens (*Gallus gallus domesticus* Linnaeus 1758) were raised from the age of 1 day in a temperature-controlled environment and later housed in separate 20 m^2 rooms at room temperature (20–23°C), and under a constant 12 h:12 h light:dark photoperiod. Animals were able to move freely and use roosts. Experiments were done in two successive stages: stage 1 and stage 2. At stage 1, the sexually mature, adult animals were fed *ad libitum* with food that contained Ca^{2+} at 3.5% of the total amount (dry weight) of nutrients. At stage 2, reduction of the available amount of Ca^{2+} in the gut (Ca^{2+} input) was accomplished in both sexes by changing to a low-calcium diet of 0.1% (dry weight). At stage 1, both sexes had reached their maximum body mass (BM), which was 1.86±0.142 kg in females and 2.62±0.135 kg in males. At stage 2, females came off lay and their BM decreased by approximately 360 g, whereas BM remained unaltered in males. At all times, chickens were provided with oyster shells *ad libitum* as a recognizable calcium source.

Sample collection

Skin samples, suction blister fluid (SBF) and blood were collected under injectional anaesthesia. The chickens were given intramuscular ketamine (Ketalar®, Pfizer, Espoo, Finland) in combination with xylazine (Rompun®, Bayer, Leverkusen, Germany) in doses of 20 mg kg^{-1} and 5 mg kg^{-1}, respectively. SBF was collected by the method first developed for humans by Kiistala and Mustakallio (Kiistala and Mustakallio, 1964) and modified for birds by Peltonen et al. (Peltonen et al., 2006). The suction cups were attached on the thoracic apterial skin. Whole blood and serum samples were taken from the brachial vein by aspiration into 2 ml lithium heparin syringes (Pico70, Radiometer Medical A/S, Copenhagen, Denmark). Blood samples for serum were removed to 500 µl vials through a short 21-22G needle. Serum was separated and collected after 1–2 h of incubation at room temperature and centrifugation.

Fluorescence labelling of DHPRs and RyRs

Frozen skin samples treated with 2% paraformaldehyde and 2% glutaraldehyde were cut into 8 µm cryosections (N=8 per sample) at –20°C and incubated in 20 nmol l^{-1} high-affinity (–)-enantiomer of dihydropyridine labelled with orange fluorophore and 0.5 µmol l^{-1} high-affinity (–)-enantiomer of ryanodine labelled with green fluorophore (Molecular Probes, Leiden, Netherlands) for 90 min, and processed as previously described by Mänttäri et al. (Mänttäri et al., 2001). The control samples were preincubated for 10 min in 10 µmol l^{-1} of the DHPR blocker nifedipine (Sigma, St Louis, MO, USA) and 50 µmol l^{-1} of the RyR blocker dandrolene (Sigma) prior to addition of labelling solution. Images of the sections were obtained with a confocal laser scanning microscope (LSM-5 Pascal, Zeiss, Jena, Germany) by using excitation at 543 nm for DHPRs and 488 nm for RyRs.

Fluorescence labelling of membrane-bound calcium

The distribution and the relative amount of membrane-bound calcium in the skin were determined in five chickens (N=5) with a fluorescent Ca^{2+}-sensitive probe, chlorotetracycline (CTC). Samples were fixed in an ice-cold mixture of 2% paraformaldehyde and 2% glutaraldehyde, 90 mmol l^{-1} potassium oxalate and 1.4% sucrose in 0.1 mol l^{-1} sodium cacodylate buffer at pH 7.4. After rinsing in phosphate buffer (pH 7.4), the samples were immersed in 2% potassium pyroantimonate/potassium hexahydroxyantimonate at 4°C. After rinsing three times for 10 min with alkaline distilled water (pH 10), samples were frozen in liquid nitrogen. Cryosectioned samples (4 µm) were treated with 100 µmol l^{-1} CTC. The CTC fluorescence was determined against the quenched fluorescence of samples treated with 10 mmol l^{-1} of the calcium-chelating agent EGTA. Samples were examined with an Olympus fluorescence microscope (Olympus, Tokyo, Japan).

Biochemical analyses

The concentration of ionized Ca^{2+} in adult chickens (stage 1) was measured with ISE (ion-selective electrodes) in fresh whole blood and in SBF as previously described by Peltonen et al. (Peltonen et al., 2006). In short, to maintain pH and ion concentration stability, fresh blood samples were stored in an ice bath without exposure to air until measured, within 2–4 h (Cao et al., 2001). SBF samples were also stored in an ice bath, but in 250 µl Eppendorf vials with an air space. Samples were analysed with a KONE Microlyte 3 device (Thermo Electron Inc., Clinical Chemistry and Automation Systems, Vantaa, Finland). In non-layers and coeval males (stage 2), we used our previous results for reference (Peltonen et al., 2006).

Plasma and SBF activity of ALP were measured by Spotchem II analysing system (Arkray Inc., Kyoto, Japan).

Statistics

One-way analyses of variance (ANOVA) were used for statistical analyses. As a *post hoc* test, Bonferroni's test or Tukey's test for multiple comparisons was used. The threshold *P* value for statistical significance was set at 0.05.

RESULTS

Localization and distribution of DHPRs and RyRs

The density of DHPRs in the skin of adult female and male chickens was determined by labelling the ion-conducting α_1-subunit with a fluorescently labelled high-affinity enantiomer of dihydropyridine. DHPRs were localized throughout the epidermis and in the dermal blood vessels (Figs 1 and 2). However, the highest densities were measured at the outermost living cell layer and at the SC (Fig. 1A,C,E). The total skin fluorescence was higher in males than in laying females, indicating a higher density of DHPRs in the male skin ($P<0.001$, Fig. 1A). After reduction of the dietary Ca^{2+} input, male density values decreased down to the level of females' in the viable epidermis and dermis, and below the female values in the SC (Fig. 2A). In females, the selective fluorescence for DHPRs was unaffected by the nutritional Ca^{2+} input or the cessation of laying (Fig. 1A, Fig. 2A).

Similar to DHPRs, the highest fluorescence intensity of labelled RyRs was in the skin surface. However, RyRs were distributed more evenly within the epidermal layers (Fig. 1E,F, Fig. 2E,F). In the mid-epidermis, punctate and intense fluorescence was clustered in proximity to the apical and basal plasma membrane. These horizontal lines were interrupted by flattened nuclei that were often found vertically aligned (Fig. 1F). In the deeper layers, high intensities were observed on the basal plasma membrane of the basal cells and on the perinuclear endoplasmic reticulum (Fig. 2F). Semi-quantitative analysis showed that the total fluorescence intensity did not differ between sexes. However, there were differences among cell layers; the living epidermis and the upper dermis showed higher intensities in males on the basic calcium diet (Fig. 1B; $P<0.001$ and $P<0.01$, respectively). With the low-calcium diet, a marked decrease in the fluorescence intensity was observed in males (Fig. 2B).

The corresponding control samples, incubated with nifedipine or dandrolene, resulted in a loss of staining in all skin layers (Fig. 3).

Ionic calcium and alkaline phosphatase in blood and SBF

The amount of biologically active Ca^{2+} in blood was the same in females and males irrespective of the physiological status associated with laying or the relative amount of calcium in the food (Fig. 4). The average Ca^{2+} concentration was 1.46 ± 0.02 mmol l^{-1}. In contrast to its stability in blood, the amount of ionic Ca^{2+} in the tissue fluid increased significantly in females after the nutritional Ca^{2+} input

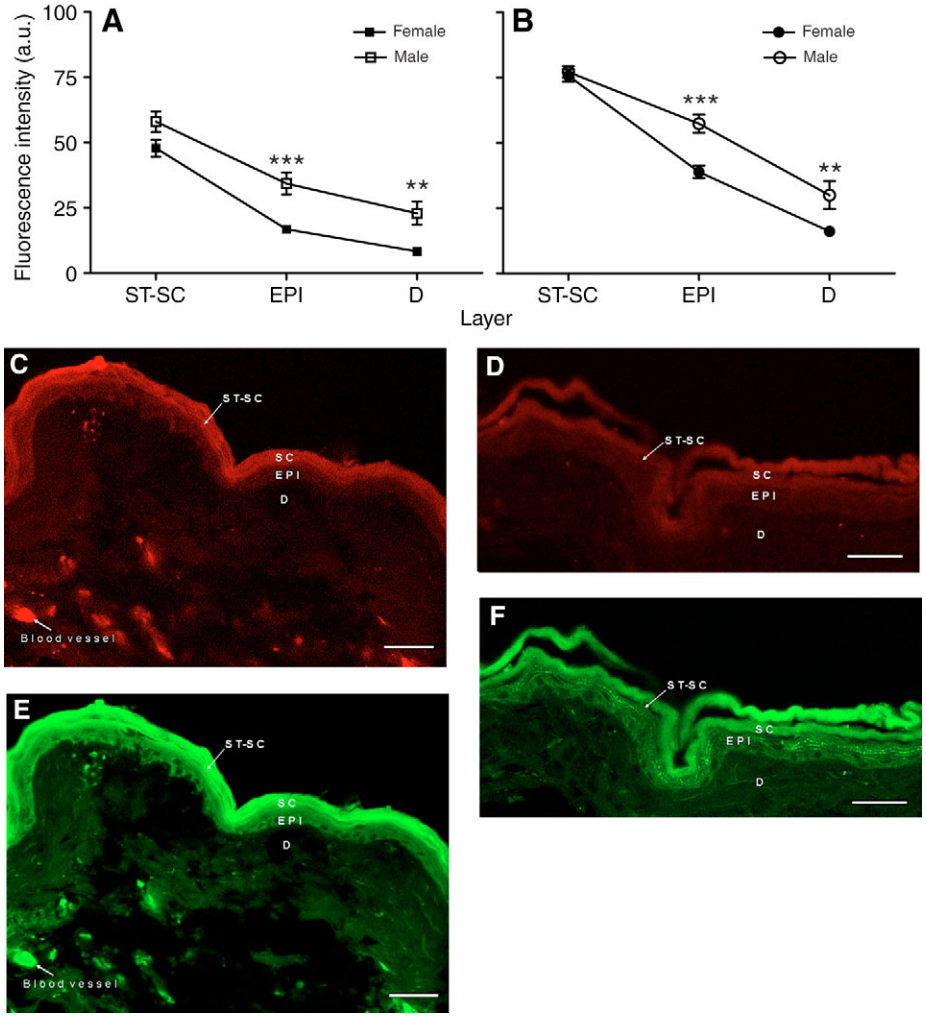

Fig. 1. Densities and distribution of dihydropyridine receptors (DHPRs) and ryanodine receptors (RyRs) in the skin of coeval adult female (layers) and male chickens. (A) Fluorescence intensities (a.u., arbitrary units) in different skin layers after incubation with a high-affinity (–)-enantiomer of DHP. (B) Fluorescence intensities in different skin layers after incubation in high-affinity (–)-enantiomer of ryanodine. (C) Distribution of DHPRs in the skin of one laying female. (D) Distribution of DHPRs in the skin of one male chicken. (E) Distribution of RyRs in the skin of one laying female. (F) Distribution of RyRs in the skin of one male chicken. Error bars denote the standard error of the mean (s.e.m.); ST-SC, innermost stratum corneum (SC) and the interface between the stratum transitivum (ST) and SC; EPI, mid-epidermis; D, outermost dermis. $^{**}P<0.01$, $^{***}P<0.001$, *post hoc* tests. Bar, 50 μm.

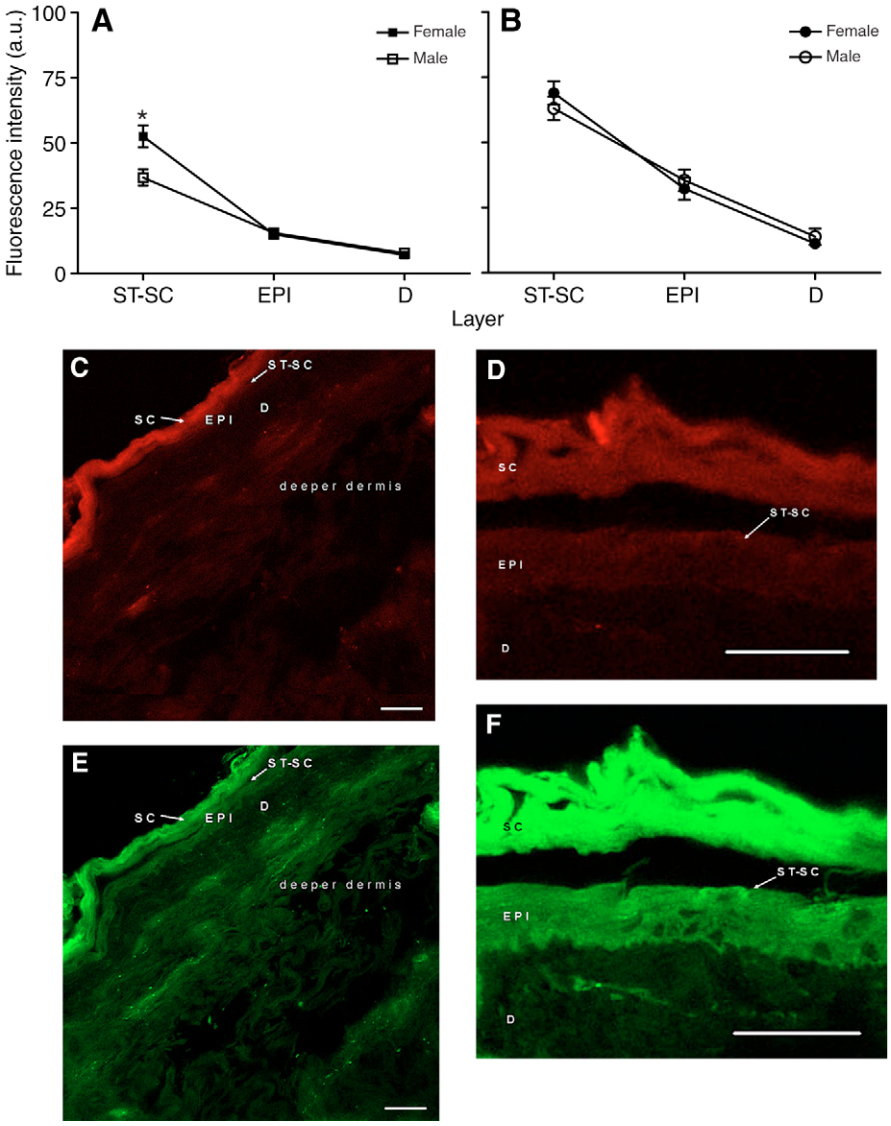

Fig. 2. Densities and distribution of DHPRs and RyRs in the skin of coeval adult female (non-layers) and male chickens on a low-Ca^{2+} diet. (A, B) Fluorescence intensities in different skin layers after incubation with high-affinity (–)-enantiomers of DHP and ryanodine, respectively. (C) Distribution of DHPRs in the skin of one female, non-laying chicken. (D) Distribution of DHPRs in the skin of one coeval male chicken. (E) Distribution of RyRs in the skin of one female, non-laying chicken. (F) Distribution of RyRs in the skin of one male chicken. Error bars denote s.e.m.; ST-SC, innermost SC and the interface between the ST and SC; EPI, mid-epidermis; D, outermost dermis. *P<0.05, *post hoc* tests. Bar, 50 μm.

decreased and they had come out of lay (Fig. 4A). In laying females, Ca^{2+} concentration was similar in SBF and blood. Males displayed higher-than-blood SBF values irrespective of the nutritional Ca^{2+} input (Fig. 4B).

The local activity of ALP was shown to be lower in the skin than in the plasma. However, this difference was statistically significant in females only (Fig. 5). After decreasing the nutritional Ca^{2+} input, a significant sex-specific difference in the plasma and SBF activities was observed; females displayed significantly higher enzyme activity than males. In three females ALP activity of the plasma was given the value of 1500 U l^{-1} since the measured activities exceeded the highest value of the linear range set for ALP activity.

Localization and distribution of membrane-bound calcium

CTC-labelled skin samples of adult male and female chickens suggest that Ca^{2+} is present in all skin layers, including SC (Fig. 6).

DISCUSSION

Our results suggest that both voltage-dependent L-type Ca^{2+} channels and RyRs exist in the skin cells of adult female and male chickens. The existence of membrane transport systems for calcium influx and intracellular release implies a more versatile functional capacity of the avian epidermis than had previously been thought. The horny layer would not be a 'dead moiety' since the Ca^{2+} entrapped within the corneocyte could be secreted *via* desquamation, or it may be secreted and recycled at the transitional interface by a secretory pathway including Ca^{2+} influx channels and peripherally located, subplasmalemmal Ca^{2+} release channels. In keeping with the physiology of the L-type channel, the epidermis may also serve as a superficial sensor for the electric potential in an epithelium practically devoid of sensory innervation (Saxod et al., 1995; Cahoon and Scott, 1999; Cahoon-Metzger et al., 2001).

Early evidence about the existence of L-type Ca^{2+} channels in the skin was obtained by Reverdin et al. (Reverdin et al., 1989) in the apical membrane of human keratinocytes by detecting inward-directed current that was activated by the DHP agonist Bay-K8644. Later, indirect evidence was obtained by using specific channel agonists, nifedipine and verapamil, which were shown to block the delaying effect of an increased extracellular concentration of Ca^{2+} on the barrier repair (Lee et al., 1992). Recently, the expression of the ion-conducting subunit of L-type channels has been demonstrated in the keratinocytes of mutant hairless mice and in cultured neonatal human keratinocytes (Denda et al., 2006). However, data on cutaneous Ca^{2+} channels are still scarce. There

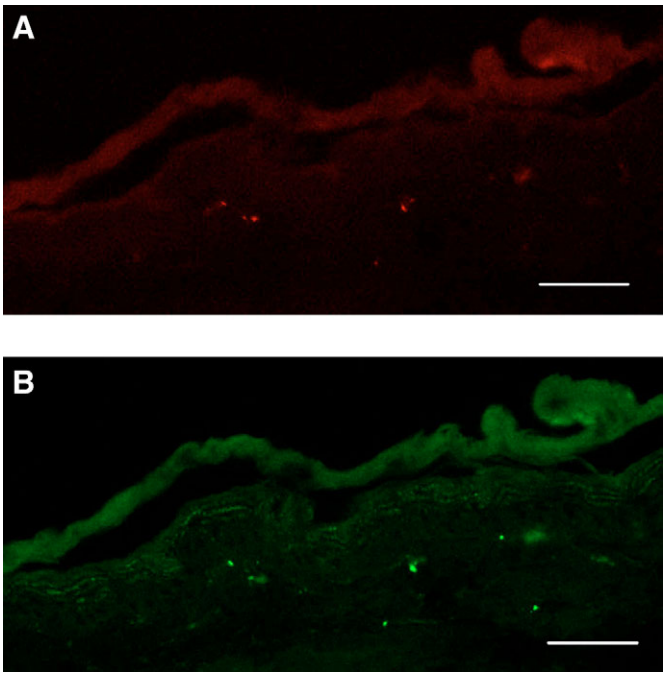

Fig. 3. Fluorescent staining of DHPRs and RyRs after blocking of the binding sites for DHP and ryanodine by the specific channel antagonists nifedipine (A) and dandrolene (B). Bar, 50 μm.

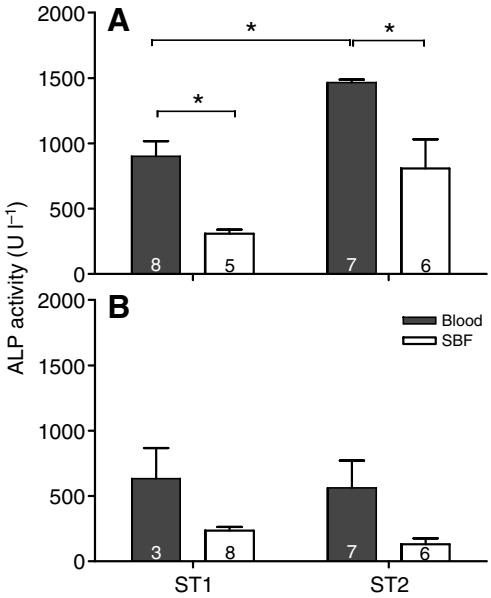

Fig. 5. ALP activity in the plasma and SBF of adult female and male chickens in different physiological stages. (A) ALP activity in female chickens. *P<0.05, *post hoc* tests. (B) ALP activity in male chickens. Error bars denote s.e.m.; ST1, adults of the same age, with females displaying regular laying cycles; ST2, adults of the same age, with females that have gone out of lay. Numbers in the columns denote N. In ST2, ALP activity in plasma and SBF was higher in females than in males (Student's unpaired t-test, P<0.001 and P<0.05, respectively).

is no evidence of the presence of RyRs in the skin, or reports on the existence of either channel in vertebrate species other than mammals. Nevertheless, the functional role of these channels in the epidermal plasticity of the skin may be very important.

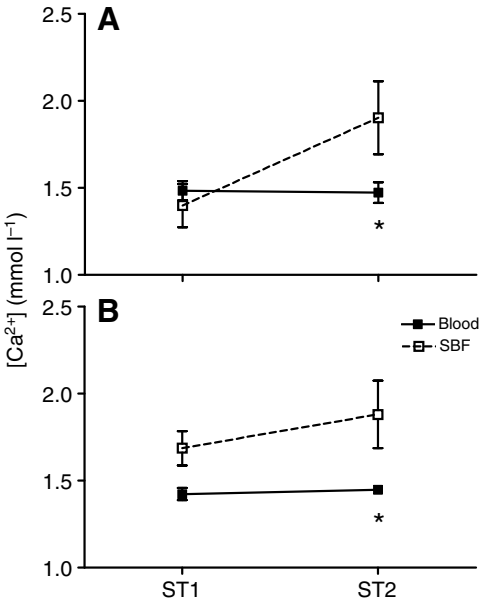

Fig. 4. Activities of ionized Ca^{2+} in the blood and suction blister fluid (SBF) of female (A) and male (B) chickens in different physiological stages. Error bars denote s.e.m.; ST1, adult females displaying regular laying cycles; ST2, adult females that have gone out of lay; *referred to in Peltonen et al. (Peltonen et al., 2006).

We hypothesized that, at the interface between the transitional and the cornified cell layer, Ca^{2+} may be secreted into the extracellular space or it may be deposited in the transitional cells and thereafter 'lost' *via* corneocyte desquamation at the surface of the skin. To support this hypothesis, our results show that the density of DHPRs and RyRs increased at the transition interface, and the density in males was higher when their nutritional Ca^{2+} input was the same as in egg-producing females. Thus, a higher DHPR and RyR density, together with a higher SBF Ca^{2+} concentration ($[Ca^{2+}]_{SBF}$), implies enhanced activity of the skin cells for Ca^{2+} influx and intracellular Ca^{2+} release. However, the amount of free Ca^{2+} in extracellular fluid *per se* does not seem to affect receptor density; a considerable decrease in nutritional Ca^{2+} input resulted in an increase in female $[Ca^{2+}]_{SBF}$ without an effect on receptor density (Fig. 2, Fig. 4A), and a sustained higher-than-blood $[Ca^{2+}]_{SBF}$ in males was associated with a lowered receptor density when nutritional Ca^{2+} input was decreased (Fig. 2 and Fig. 4B).

The expression of DHPR and RyR genes is not tissue specific, even though they are predominantly expressed and their functional connection is best described in skeletal, cardiac and smooth muscle cells (Franzini-Armstrong, 2004). Until the present study, DHPRs had not been shown to coexist with RyRs in non-excitable epithelial cells. Previously, DHPRs have been visualized in the epidermis of mice and neonatal humans (Denda et al., 2006), and their functionality has been demonstrated in the pigment epithelium of the retina in rats, monkeys and humans (Ueda and Steinberg, 1993; Ueda and Steinberg, 1995; Mergler and Strauss, 2002). Expression of RyRs has been shown in the epithelial cells of the cornea (Socci et al., 1993) and in the epithelial cells of the kidney tubulus (Tunwell and Lai, 1996). Together, previous and our present findings suggest that intracellular signalling *via* RyRs is not exclusively confined to excitable cells, and that DHPRs and

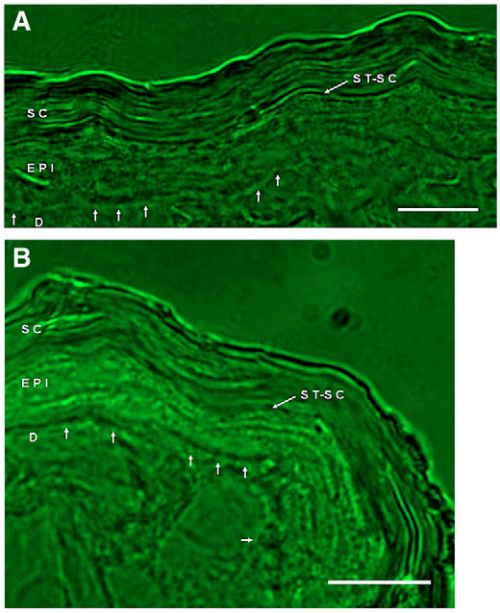

Fig. 6. Distribution of membrane-bound Ca^{2+} in different skin layers of adult chickens. (A) Specific fluorescence, obtained with the fluorescent Ca^{2+}-sensitive probe chlorotetracycline, in one laying female. (B) Distribution of Ca^{2+} in different skin layers of one male chicken. ST-SC, innermost SC and the interface between the ST and SC; EPI, mid-epidermis; D, outermost dermis. Short arrows denote the basal lamina. Bar, 10 μm.

RyRs might be spatially and functionally connected in non-excitable skin cells.

According to our results in chickens, the possible channel units of DHPRs and RyRs in a sebokeratinocyte are peripherally located. This spatial relationship seems to resemble the arrangement of the smooth muscle cell in which the sarcoplasmic proteins, calsequestrin and RyRs colocalize with DHPRs in numerous, peripherally located sites within the caveolar domains (Moore et al., 2004; Pucovsky and Bolton, 2006). Due to the native arrangement of the stratified epidermis in our study, the exact array of DHPRs on the plasma membrane could not be revealed. However, RyRs were located in the proximity of the plasma membrane in horizontally aligned clusters, indicating the possible sites where the two channels might interact *via* spatial proximity. In a single smooth muscle cell of the urinary bladder, DHPRs have been shown to occupy the plasmalemma in longitudinal stripes that overlap almost entirely with the corresponding stripes formed by labelled RyR proteins (Moore et al., 2004). The authors estimated that a single cell may display approximately 100–200 overlapping patches, i.e. sites where the transduction of membrane depolarization into the release of Ca^{2+} from the sarcoplasmic reticulum (SR) takes place. In avians, cardiac cells also seem to display the pattern of peripheral location of DHPR/RyR units. Excitation–contraction units develop *via* docking of the SR membrane to the surface membrane due to the lack of intruding T-tubules in these cells; the appropriate locations and quantities of DHPRs and RyRs within the unit are established gradually during development (Sun et al., 1995; Protasi et al., 1996).

The avian epidermis is formed by columns of non-excitable epithelial cells that move and differentiate *via* a vertical pathway from the basement membrane to the surface of the skin. Its sensory functions seem to be limited since it is inhibitory, or at least non-permissive, to the growth of sensory nerves (Saxod et al., 1995; Cahoon and Scott, 1999; Cahoon-Metzger et al., 2001), and its outermost layer is generally categorized as a dead moiety of the skin. Nevertheless, according to our findings, the strongest specific fluorescence for the DHPRs is located in the surface of the skin, implying a voltage-sensing function. Previously, maintaining the surface electric potential at a negative value of about –3 mV was shown to be essential for the homeostasis of the skin in mice (Denda et al., 2001). It was suggested that L-type Ca^{2+} channels might be involved because specific channel blockers for the α_1-subunit helped to restore the ionic balance when it was disrupted (Denda et al., 2006).

The ionic environment of the avian epidermis might differ considerably from that of mammals. In pigeons (*Columba livia* Gmelin 1789), Ca^{2+} has been shown to accumulate in the corneocytes at the transitional interface and remain in the SC up to the outermost cell layers (Peltonen et al., 2003). Our preliminary results obtained using CTC, the fluorescent marker of calcium, support the idea that calcium is also preserved in the chicken SC and might colocalize with DHPRs and RyRs in the epidermis. Given that the composition of the extracellular fluid affects the function of channels sensitive to voltage, an increased concentration of monovalent cations outside the cell is likely to alter the open–closed probability of these channels, and thus the cellular response. To date, only little is known about the character, location and magnitude of the L-type Ca^{2+} currents in skin cells.

We measured plasma and SBF activity of ALP in order to estimate the level of ALP-mediated Ca^{2+} utilization in adult female and male chickens. Since it is known that bone remodelling is accompanied by local fluctuations of free extracellular Ca^{2+} concentration (Parfitt, 1987), we expected to see changes in the activity of ALP in relation to nutritional Ca^{2+} input and Ca^{2+} concentration. Our results show that there was a clear sex-specific difference in the response to the reduced nutritional Ca^{2+} input: the plasma and skin activity of ALP increased in females in association with the increased Ca^{2+} concentration of SBF but not blood, whereas in males the activity of ALP was unaffected by the level of nutritional Ca^{2+} input and $[Ca^{2+}]_{SBF}$ values were displayed at a sustained, higher-than-blood level. It is clear from these results that both sexes are able to regulate the amount of free Ca^{2+} in blood at a normal level despite the change in diet. Because the chickens were feeding freely, the extent of the possible compensation for low-calcium feed by oyster shells remains unknown. However, the reduction in Ca^{2+} input was functionally effective since all females went out of lay. Together, our findings on ALP activity suggest that either physiological or pathological ALP-mediated mineralization could take place in the skin. These processes are most probably confined to the dermis because the activity measured in the epidermis was found to be less than 1% of the activity of the underlying dermis (Mier and van Rennes, 1982). In general, the cutaneous ALP seems to be of a tissue-non-specific type, present in the dermal condensations of developing feather germs, hair follicles, capillaries and sweat glands (Mier and van Rennes, 1982; Crawford et al., 1995; Saga and Morimoto, 1995). Furthermore, it is present in both a soluble and a membrane-bound form (Mier and van Rennes, 1982). In the present study, the cellular location of ALP activity could not be assessed. However, feather follicles and sweat glands are excluded as the sites of enzyme activity because the experimental area is void of these structures.

Since the skeletal growth was already completed in both sexes, we expected that fluctuations in the plasma activities of ALP would be sex specific and probably associated with egg shell calcification.

This suggestion of the association between ALP activity and egg shell calcification has been put forward by several authors (Wilcox et al., 1963; Singh et al., 1983; Carpenter et al., 2001; Harr, 2002), but opposed by others (Arad et al., 1989; al-Bustany et al., 1998). Our findings show no difference in plasma activity between laying females and males of the same age. Thus, our results are in line with studies indicating that the plasma activity of ALP is not associated with egg shell calcification but, instead, with the physiological state that ensues at the cessation of laying. The cause of this increased ALP activity in both plasma and SBF is not known. In avians, increases in the plasma level of ALP activity appear to be associated with osteoblast or bone-forming activity. Such activity is present especially during skeletal growth after hatching (Campbell and Coles, 1986; Vinuela et al., 1991; Lumeij, 1997; Tilgar et al., 2004a). In the present study, skeletal growth was complete, excluding somatic growth as a causative factor. After skeletal growth is complete, total ALP activity, and especially the activity of its bone-derived isoform (Tilgar et al., 2004a; Tilgar et al., 2004b), decreases, given that the amount of calcium is adequate for normal growth (Meluzzi et al., 1992; al-Bustany et al., 1998; Jurani et al., 2004). Since increased ALP activity has also been associated with osteoblast activity in general, the observed increase in the females that have gone out of lay might be caused by the reversal of the formation of the medullary and structural bone. When laying ceases, medullary bone disappears and the formation of structural bone recommences accompanied by increased osteoblast activity (Whitehead, 2004).

In conclusion, the presence of L-type Ca^{2+} channels for ion influx and RyRs for Ca^{2+} release in the skin cells of female and male chickens suggests that their skin has the functional capacity for accumulation, storage and secretion of Ca^{2+}. Thus, the higher density of these channels in males receiving excessive Ca^{2+} implies an increased capacity for Ca^{2+} influx and intracellular processing. Whether there are structural and functional interactions between DHPRs and RyRs in the sebokeratinocytes is not known. However, the peripheral location and the high receptor density at the site of exocytosis of the lamellar bodies in the interface between the transitional and horny layers imply a role in a signalling pathway for secretion. Together, the horny layer seems to be more a live than a dead moiety of the skin! Variations in the ALP activity suggest that Ca^{2+} is utilized in the skin. In females, the increased activity may be connected with the reformation of structural bone after the cessation of laying.

We wish to thank the Animal Care and Management unit and the Department of Basic Veterinary Sciences of the Faculty of Veterinary Medicine, University of Helsinki, for providing facilities. We also thank M. Sc. Katja Anttila of University of Oulu, for technical support.

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