Localization of Plasma Membrane Calcium ATPase 2 and 4 (PMCA2 and PMCA4) in Chicken Sperm

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Abstract. Calcium ions play important roles in several cellular processes including signalling pathway, membrane fusion and cell adhesion. In sperm, it is known that intracellular calcium concentration ([Ca²⁺]) regulates acrosome exocytosis and flagellar motility. [Ca²⁺], is generally regulated by influx and efflux of calcium ion. Plasma membrane calcium ATPase (PMCA) pumps the [Ca²⁺], excess, providing a fine-tuning of [Ca²⁺], concentration for sperm functions. To date, information related PMCA family in chicken sperm is still not documented yet. Therefore, this study was subjected to characterize expression and localization of PMCA family in chicken sperm. In this study, we selected PMCA2 and PMCA4 as representative to a specific tissue and a housekeeping isofrom, respectively. Immunohistochemistry of PMCA2 and PMCA4 in chicken testis showed that both transmembrane proteins are expressed at the edge adluminal of the seminiferous tubule, indicating that these proteins are present in morphologically matured sperm that completed spermatogenesis. This was confirmed by immunocytochemistry, showing that PMCA2 and PMCA4 are highly enriched in the midpiece of chicken sperm. Furthermore, sub-cellular fractionation, combined with western blotting, showed that both isoforms are exclusively found in the membrane fraction of the sperm, together suggesting both PMCA are localized to the plasma membrane overlaying midpiece.

1. Introduction

According to the Food and Agriculture Organisation’s (FAO) statistics, the global demand for poultry meat and eggs tend to increase in the next decades exponentially as a result of population growth and changes in dietary habits. Total poultry meat demand in developing countries is projected to double (or approx. 93.5 million tonnes) by 2030. This is a challenge for our ability to develop the livestock production efficiently.

To date, many efforts have been made to develop chicken production systems to maximise production ability. Recent developments in the field of poultry reproductive technology have led to the importance of fertility as a main chord in sustainable chicken production. For example, sperm cryopreservation and artificial insemination have been widely used for genetic make-up and efficiency use of the male genetic resource. However, a significant problem with this kind of applications is the fertility rates remain unreliable for use in commercial production. This is mostly because it is still unclear the natural state of mechanisms by which chicken sperm undergo deterioration of fertilizing ability underlying sperm preservation.

For the successful fertilisation, sperm require to travel in the female reproductive tract and undergo physiological changes before reaching the female egg. Moreover, after reaching the target, the sperm must interact with the egg’s extracellular matrix and perform acrosomal exocytosis. Calcium is considered playing roles in most of these processes. It is well known that the elevation of intracellular calcium ([Ca²⁺]) plays a vital role as a second messenger in the physiology of cells. In sperm, [Ca²⁺].
responsible for flagellar movement as well as activation of signalling pathways leading to acrosome exocytosis that pre-requisite to fertilise the oocyte [1]. Previous studies documented that the increase of basal \([\text{Ca}^{2+}]\) level ranged from 25-75 nM to 500 nM leads to membrane fusion and acrosome reaction [2,3]. The functional importance of \([\text{Ca}^{2+}]\), is also supported by the evidence that the fertilising ability of cryopreserved mammalian sperm is impaired by extreme elevation of \([\text{Ca}^{2+}]\), due to the compositional changes in sperm membrane during cryopreservation [4,5]. Even though the elevation of \([\text{Ca}^{2+}]\), is essential for sperm functions, but the sustained elevation of \([\text{Ca}^{2+}]\), is toxic for the cell [6].

Plasma membrane calcium ATPase (PMCA), transmembrane proteins acts as a primary channel involved in ATP-dependent expulsion \([\text{Ca}^{2+}]\), providing a fine-tuning \([\text{Ca}^{2+}]\), for sperm functions. PMCA consists of four different isoforms, where PMCA1 and PMCA4 are expressed in several tissues including testis, while PMCA2 and PMCA3 are expressed in very limited tissues [7], suggesting the functional distinction between isoforms. In fact, PMCA1 and PMCA4 are highly conserved in sperm from different species [7,8]. In murine sperm, PMCA4 has been found in sperm tail [9], while in bull sperm it is enriched in the midpiece [10]. Although some researches have been carried on the functional importance of PMCA in sperm from different animals, no single study exists which focus on avian sperm. Therefore, this study investigates the subcellular location of PMCA2 and PMCA4 as a foundation to dissect regulating mechanisms of \([\text{Ca}^{2+}]\), in chicken sperm.

2. Materials and Methods

2.1. Localisation of PMCA2 and PMCA4 in chicken testis

We firstly deparaffinized and rehydrated paraffin embedded testis in 100% xylene, 100% ethanol, and 70% ethanol for 15, two, and one minute, respectively. Heat-induced epitope retrieval for 10 minutes in 0.01 M citrate buffer, pH 6.0 was performed and followed by cooling down at room temperature (RT). Blocking solution (10% of goat serum in PBS-) incubated the slides for one hour at RT. The slides were then incubated in PMCA2 or PMCA4 polyclonal antibody [1:50 rabbit serum immunoglobulin (IgG) antibody in PBS-)] at 4 °C for overnight. After three times washing in PBS-, anti-rabbit IgG Alexa Fluor 488 antibody (1:200) was applied to incubate the slides for one and half hour at RT. Coverslips were mounted on to the glass slides using VECTASHIELD mounting medium with 4’-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). Histological sections of each sample were used as negative controls, substituting primary antibody with PBS-. All procedures after antigen retrieval were performed in a humid chamber to avoid the slides to dry out.

2.2. Localization of PMCA2 and PMCA4 in chicken sperm

Sperm were allowed to settle on anti-liquid bordered coverslips at 39 °C for 30 minutes, followed by fixation in 4% paraformaldehyde for 15 minutes at RT. The sperm samples were then washed, and permeabilized in 0.5% Triton X-100 for 1 min. Blocking solution (10% goat serum in PBS-) was applied on to the samples for 1 hour at RT. The samples were then incubated in a primary antibody (chicken PMCA2/PMCA4 polyclonal antibody 1:150 PBS-) at 4 °C for overnight. After three times washing in PBS-, the samples were then incubated in goat anti-rabbit IgG Fluor 488 antibody (1:200). VECTASHIELD mounting medium with 4’6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) mounted the coverslips on to glass slides. Sperm samples without a primary antibody were used as negative controls. All the procedures were performed in a humid chamber.

2.3. Sub-cellular fractionation of PMCA2 and PMCA4 in chicken sperm

Sub-cellular fractions of sperm were prepared from chicken sperm according to [11], with slight modifications. In brief, sperm samples were washed by PBS- for 5 minutes at 1000 x g. The resulting pellet was then resuspended in PBS- containing protease inhibitors (Free EDTA protease inhibitors cocktail, Roche, Basel, Switzerland) and 0.5% Triton X-100. The supernatant was then homogenised, five
times burst sonicated, and centrifuged at 4 °C, 10000 x g, for 10 minutes. The resulting pellet was designed as a “cytoskeleton” fraction; the supernatant was then centrifuged at 4 °C, 20000 x g, for two hours. The pellet and supernatant were subjected as “membrane (Me)” and “cytosol (Cs)” fractions, respectively. All the sperm fractions were then quantified using Bradford Assay Kit (Bio Rad, US). 70 µg of protein from each fraction was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

3. Results and Discussion

3.1. Localisation of PMCA2 and PMCA4 in chicken testis

PMCA is known as a transmembrane protein which has the main task in Ca\(^{2+}\) clearance [12]. The protein consists of four isoforms, PMCA1-4, encoded by four different genes and have tissue-specific expression. PMCA1 and 4 are expressed in most tissues and act as housekeeping isoform, whereas PMCA2 and 3 are more restricted, mostly in brain and striated muscle [13]. Okunade et al. (2004) reported that PMCA4 is more abundant isoform in mouse testes compared to PMCA1, making PMCA4 is the major isoform in the tissue, even though PMCA1 can compensate its absence. In contrast to housekeeping isoforms, a previous study observed in mice that PMCA2 acts as the primary controller of milk calcium concentration as 60% of milk calcium decline is due to the absence of PMCA2 [14]. Moreover, the study also revealed that the null PMCA2 mutation of mice cannot be fully compensated by other calcium-transporting channels, secretory pathway Ca\(^{2+}\)-ATPase (SPCA) and sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA). Considering the functional importance of PMCA2 and PMCA4 in regulating [Ca\(^{2+}\)], these isoforms might play roles in chicken sperm functions, since the sperm require calcium-dependent events to acquire their fertilizing abilities. However, no previous study has investigated PMCA2 and PMCA4 in chicken sperm, making calcium mechanisms underlying sperm functions are still unclear. Therefore, this study is firstly subjected to identify the localisation of PMCA2 and PMCA4 in chicken sperm to gain further insights regarding the specific biological functions of calcium-dependent sperm functions.

In this study, two different polyclonal primary antibodies-against chicken PMCA2 and PMCA4 were used to determine the origin of the proteins in chicken testes. Both proteins were detected in chicken seminiferous tubules from the basal to the edge adluminal compartment of seminiferous tubules (Fig.1). This localisation indicates that both PMCA2 and PMCA4 might be synthesised and embedded into sperm membrane during spermatogenesis. The similarities between PMCA2 and PMCA4 protein localization indicate the requirement of Ca\(^{2+}\) clearance at late spermatogenic cells. The fluorescent intensity emitted from the edge of adluminal compartment is stronger than that from the basal compartment. A possible explanation for the stronger intensity at the edge adluminal seminiferous tubules was that the cytosolic content in mature sperm is less than non-mature sperm. At this compartment, the spermatids undergo extensive remodeling, whereby their cytoplasm content is removed by approximately 70% during spermiation [15,16], resulting in the cell more compact and dense. Moreover, numerous proteins have also been localized to specific sites during spermiation [17].

3.2. Localisation of PMCA2 and PMCA4 in chicken sperm

In order to investigate the distribution of PMCA2 and PMCA4 in particular in subcellular compartment of chicken sperm, immunocytochemistry on chicken sperm was performed. Our results identify that chicken sperm express the family members of plasma membrane calcium ATPase, PMCA2 and PMCA4 (Fig. 2). These both proteins are localized on the specific subcellular compartment at the level of the midpiece. This localization is the same with that in bull sperm [10], and is direct vicinity to the high concentration Ca\(^{2+}\) stores in mammalian sperm [18]. In the midpiece of sperm where mitochondria are abundant, the production of ATP is generated by an increase in [Ca\(^{2+}\)], via Ca\(^{2+}\) sensitive dehydrogenases and NADH production [19].
It has been known that the increase of \([Ca^{2+}]_i\) is essential for sperm to acquire the fertilizing abilities. For example, it plays roles in sperm capacitation in bull [20], acrosome reaction in bull and ram [21], and sperm-oocyte binding [22]. However, the sustained high level of \([Ca^{2+}]_i\) leads to coagulative necrosis due to the loss of plasma membrane’s ability to maintain a large gradient of calcium ions [6]. PMCA family acts as \([Ca^{2+}]_i\) pumps to expel excess \([Ca^{2+}]_i\) which makes sperm to excite fertilization ability collectly. This was proved by a previous study using mutant mice showing that deletion of PMCA4 results in double \([Ca^{2+}]_i\) concentration [9] and failure of sperm capacitation and hyperactivated motility [8]. Although the functional relevance of PMCA2 and PMCA4 in chicken sperm have not been established, it is attempting to predict that the PMCA family members located in the midpiece are responsible for \([Ca^{2+}]_i\) maintenance underlying sperm functions, in particular in the flagellar movement.

**Figure 1.** Immunohistochemistry in paraffin-embedded chicken testes using polyclonal antibody against PMCA2 and PMCA4. Immuno-positive signals of both PMCA2 and PMCA4 were detected at the basal to the edge of adluminal

**Figure 2.** Immunocytochemistry of PMCA2 and PMCA4 in chicken sperm. PMCA2 and PMCA4 were identified in the midpiece of the sperm (green). Nuclei were fluorescence-labelled using DAPI (blue). Bars: 5 µm.
3.3. Sub-cellular fractionation of PMCA2 and PMCA4 in chicken sperm

Membrane fraction of chicken sperm was prepared using Triton X-100, a non-ionic detergent. Western blotting analysis was performed using a polyclonal antibody against each for PMCA2 and PMCA4. PMCA2 and 4 showed their distribution into both cytosol and membrane fractions. This is consistent with a previous study showing the abundance of PMCA4 in membrane fraction isolated from bull sperm [10].

Lipid rafts are specific membrane domains that play an important roles in diverse cellular processes. In general, PMCA tends to be concentrated into caveolae, a particular type of lipid rafts compartment to govern various signal transduction pathways [23]. This type of lipid rafts is surrounded and enriched by acidic phospholipids such as cholesterol (CHOL), sphingomyelin (SPM), phosphatidic acid (PA), phosphatidylyserine (PS), phosphatidylinositol(PI), and phosphatidylinositol 4,5-bisphosphate (PIP$_2$) which stimulate the velocity maximum ($V_{max}$) and decrease enzyme’s affinity ($K_m$) of PMCA [24,25,26]. The stimulated-route can also be noticed from calmodulin-stimulated enzymes. The binding event of acidic phospholipid and polyunsaturated fatty acids to calmodulin and the first cytosolic loop of PMCA activate the pump to decrease [Ca$^{2+}$]$_i$ by 100 nM [27,28]. Intrestingly, recent our study demonstrated that chicken sperm possess lipid rafts in sperm head region same as where localization of PMCA2 and 4 are found in chicken sperm, suggeting future investigation for a potential mechanism for regulation of PMCA function in sperm.

Figure 3. SDS-PAGE and Western blotting analysis of PMCA2 and 4 in chicken sperm membrane fractions. Protein (70 µg per lane) were probed with polyclonal antibody against chicken PMCA2 and 4. PMCA2 and 4 were localized in cytosol (Cs) and membrane (Me) fractions. Cytoskeleton fraction is abbreviated as CSK.

Evidence presented in this paper indicates potential important of PMCA2 and 4 maintaining the basal [Ca$^{2+}$]$_i$ and provides a foundation to dissect a molecular mechanism involved in regulation of flagellar motility. In addition, it has been shown that lipid rafts have direct interaction with a number of Ca$^{2+}$-activated signalling molecules in caveolae, such as protein kinase C (PKC) and phosphatidylinositol 4,5-bisphosphate (PIP$_2$) [29,30], which suggest potential roles of PMCA in sperm functions. Therefore, this study needs to address identification of the signaling molecules regulated by lipid-protein interaction to unravel the downstream events of PMCA regulating [Ca$^{2+}$]$_i$ for chicken sperm functions.

4. Conclusion

The localization of PMCA2 and PMCA4 in chicken testes and spermatozoa have similar localization which were strongly expressed at the edge of adluminal seminiferous tubules and the midpiece,
respectively. Also, our findings identified the subcellular distribution of these [Ca\textsuperscript{2+}] pumps were found in both the cytosol and membrane.

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