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Expression of phosphatidylcholine biosynthetic enzymes during early embryogenesis in the amphibian *Bufo arenarum*

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Key words: CTP:phosphocholine cytidylyltransferase, choline kinase, embryo development, amphibians.

Running title: Phosphatidylcholine biosynthesis in development.

Abstract

In the principal route of phosphatidylcholine (PC) synthesis the regulatory steps are catalyzed by CTP:phosphocholine cytidylyltransferase (CCT) and choline kinase (CK). *Knock-out* mice in *Pcyt1a* (CCT gene) and *Chka1* (CK gene) resulted in pre-implantation embryonic lethality, demonstrating the essential role of this pathway. However, there is still a lack of detailed CCT and CK expression analysis during development. Our aim was to study the expression during early development of both enzymes in the external-fertilization vertebrate *Bufo arenarum*. RT-PCR and western blots confirmed their presence in unfertilized eggs. Analysis performed in total extracts from staged embryos showed constant protein levels of both enzymes until the 32-cells stage. Then, they decreased reaching a minimum in gastrula after which they started recovering. CCT is an amphitropic enzyme that interconverts between cytosolic inactive and membrane-bound active forms. Immunoblot analysis demonstrated that cytosolic/total CCT protein ratio does not change along embryogenesis, suggesting a progressive decline of CCT activity in early development. However, PC (and phosphatidylethanolamine) content per egg/embryo remained constant throughout the analyzed stages. In conclusion, our data in *Bufo arenarum* suggest that net synthesis of PC mediated by CCT and CK is not required in early development and that supplies for membrane biosynthesis are fulfilled by lipids already present in the egg/embryo reservoirs.

Introduction

Phosphatidylcholine (PC) is the most abundant phospholipid in mammalian cells and plays an important structural role in cellular membranes (Jackowski and Fagone, 2005; Vance and Vance, 2004). The CDP-choline pathway (Kennedy pathway) is the principal route for PC biosynthesis involving three steps; choline is an essential nutrient in mammals and is transported into the cell and phosphorylated by choline kinase (CK). CTP:phosphocholine cytidylyltransferase (CCT) catalyzes the condensation of CTP and phosphocholine to form CDP-choline, which in turn donates the phosphocholine moiety to diacylglycerol to form PC in a reaction catalyzed by CDP-choline:diacylglycerol cholinephosphotransferase (Vance and Vance, 2004). CCT mediates the rate-limiting step in this pathway (Jackowski and Fagone, 2005; Vance and Vance, 2004) and the regulation of its enzymatic activity is

characterized by its association with membrane structures (Jackowski and Fagone, 2005; Vance and Vance, 2004). A current model suggests that the bi-layer curvature elastic stress is sensed by CCT and governs the degree of membrane association, thus providing a mechanism for both positive and negative regulation of activity (Attard et al., 2000; Davies et al., 2001).

Immediately following fertilization, vertebrate eggs undergo a series of divisions pushing the embryo into a coordinated new plasma membrane biosynthesis. In general, in this first period, the embryo does not become larger and the cells become smaller with each division. Therefore, the synthesis and integrity of cellular membranes is likely to be an important controlling factor during embryogenesis of vertebrate animals (Gilbert, 2000). In particular, in mouse it was demonstrated that pre-implantation embryos are capable of synthesizing phospholipids *de novo* and equipped to assemble new intracellular membranes and plasma membrane (Pratt, 1980). Also, the specific role of *Chka* and *pCyt1a* was evaluated by generating *knock-out* (KO) models. *Chka*$^{-/-}$ mice were recovered at the blastocyst stage yet not at embryonic day 7.5 (Wu et al., 2008) and *Pcyt1a*$^{-/-}$ mice failed to form blastocysts underscoring the importance of PC and the Kennedy pathway during early development (Wang et al., 2005). Wang et al. suggested that the ability of the KO pre-implantation embryos to progress through the first few divisions is likely due to the expression of maternal CCT that is present at high levels in mature eggs (Jackowski et al., 2004).

The characteristics of the mouse model complicate the analysis of enzyme levels during early development and related experiments remain to be done. Amphibians are the eligible systems for these approaches due to advantageous external fertilization and development, abundant gametes and embryos for biochemical studies, ease of manipulation and identification of their developmental stages. Amphibians like other vertebrates with external fertilization contain yolk platelet. In particular, in the amphibian *Bufo arenarum* about the 80% of the whole embryo phospholipids were found in yolk platelet fraction and PC and phosphatidylethanolamine (PE) are the major phospholipids in these structures. Moreover, ^{32}P labelling experiments demonstrated that immediately after fertilization there is an active phospholipid metabolism (PC and PE synthesis) which continues at least until late gastrula (Alonso et al., 1982a).

In this work, we show that CCT and CK are abundantly expressed in unfertilized eggs. The levels of these enzymes decline as development progresses and reach a minimum around gastrula stage, after which they start to recover. Interestingly, the lower levels of expression mainly overlap with the mid blastula transition (MBT). The cytosolic to total CCT protein ratio is constant during all the developmental stages analyzed indicating that the activity of CCT diminishes along development until gastrula. However, the mass of PC and PE per egg/embryo is constant suggesting that in *Bufo arenarum* eggs the net synthesis of phospholipids appears to be not essential during the first developmental stages thanks to yolk lipids reservoirs present in the unfertilized eggs. To our knowledge, this is the first time that CCT and CK protein levels are studied during early development in vertebrates.

Materials and Methods

Animals, gametes and embryos.

Sexually mature *Bufo arenarum* specimens were collected in the neighbourhoods of Rosario city and kept in a moist dark chamber at 12ºC until used. All experiments were performed in conformity with the guide for the care and use of laboratory animals promulgated by the National Institute of Health (National Center for Research Resources), Bethesda, MD, USA.

Testes were dissected from male toads, and spermatozoa were obtained by mincing the organs in Ringer-Tris solution (0.11 M NaCl, 2 mM KCl, 1.4 mM $CaCl_2$, 10 mM Tris, pH 7.2) at 4ºC. The homogenate was filtered through gauze and centrifuged for 10 min at 130 *g* at 4ºC to remove blood cells and tissue debris. The sperm suspension was centrifuged for 10 min at 650 *g* at 4ºC. Pelleted spermatozoa were resuspended in Ringer-Tris solution.

Female specimens were kept in a moist chamber at 20-22ºC for 1 day before stimulation with one homologous hypophysis homogenate injected intracoelomically. After 10-12 h, strings of eggs were ovulated or collected from ovisacs.

Just before fertilization, eggs were placed into 10 mL of 10% Ringer in a Petri dish, then 100 µL of the minced testes solution (~10 million sperm) was added to the unfertilized eggs, incubated 30 min and finally washed with Ringer. These conditions allow obtaining typically a 100% fertilization success rate.

Bufo arenarum embryo stages.

Embryos were staged according to Del Conte and Sirlin (1952). At each stage studied the removal of jelly coats was accomplished by immersion of egg/embryo strings in 1% sodium thioglycolate (pH 8) solution. Dejellied eggs/embryos were thoroughly rinsed with Ringer solution, frozen in liquid nitrogen and kept at -70 ºC.

Total membranes and cytosol isolation.

Sub-cellular fractionation was performed according to the procedure described by Sato et al (1999) with modifications. All procedures were carried out at 4°C. Approximately 200 dejellied eggs/embryos were mixed with 2 ml of buffer A (20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4) supplemented with 1% protein inhibitor cocktail (Sigma) and 2 mM PMSF and homogenized in a Teflon-glass potter. The homogenate was centrifuged 10 min at 1000 *g* (SS34 Rotor, Sorvall Instruments) to remove cellular debris and yolk platelets. The supernatant (Total Extract) was collected and centrifuged at 150000 *g* for 1 h (Ti80 rotor, Beckman L-80 ultracentrifuge). After centrifugation, the clear supernatant was set aside as the Cytosolic fraction. The pellet and fluffy part of the pellet were then carefully taken, resuspended with buffer A and centrifuged at 150000 *g* for 30 min. The pellet fraction obtained was resuspended in 250 µl of buffer A plus 1% Triton X-100 and sonicated (Total Membrane fraction). By this procedure the efficiency was of 7 ± 0.4 µg of cytosolic protein/embryo and 3.8 ± 0.4 µg of membrane protein/embryo. The total amount of protein per egg/embryo was of 148.8 ± 5.8 µg. This amount and the yield of cytosolic and total membrane proteins were independent of the developmental stage.

Protein Assays.

Protein concentrations were determined according to Sedmak and Grossberg (1977), using bovine serum albumin as standard.

Electrophoresis, electrotransfer and western blot analysis.

Polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) was performed essentially according to the Laemmli's Method (1970). Samples were diluted with an appropriate volume of 6X sample buffer with 0.1 M dithiothreitol, boiled for 5 min, and loaded onto 12% acrylamide mini-gels with 5% stacking gel, and electrophoresed at 20 mA/gel (MiniProtean III Gel System, *BioRad*, Hercules, CA, USA). The apparent molecular weights were estimated with molecular mass standards (*Amersham Pharmacia Biotech*, Piscataway, NJ, USA). Gels were processed for Coomassie Brilliant Blue staining

or electrotransferred to nitrocellulose membranes according to Towbin's method (Towbin et al., 1979).

Membranes were blocked with TBS buffer supplemented with 3% w/v BSA and 0.1% v/v Tween 20. Then, membranes were washed and incubated with a 1:500 dilution of the primary antibody for 1 h (anti CCT rabbit polyclonal, gently provided by Dr. Suzanne Jackowski, Department of Infectious Diseases, St. Jude Children´s Research Hospital, Memphis, USA or anti-CK rabbit polyclonal (ChoK (H-210) *Santa Cruz Biotechnology Inc*., Dallas, TX, USA). After washing, membranes were incubated with the appropriate HRP-conjugated antibody, washed and developed using chemiluminescence and X-ray films. When membrane re-probing was necessary, 0.2 M NaOH treatment was used for stripping. Actin detection on membranes was performed to ensure equal loading. The antibody used was a rabbit polyclonal at a 1:600 dilution (sc-1616, *Santa Cruz Biotechnology, Inc*., Dallas, TX, USA) and the manufacturer's instructions were followed.

Exposed films were digitized and quantified using Gel-Pro Analyser 3.0 (Media Cybernetics). The integrated optical densities (IOD) corresponding to CK and CCT were normalized to their respective Actin IODs. In the studies dealing with CCT sub-cellular distribution, the normalized CCT values were also normalized to the total protein content of the respective fraction (cytosol or total extracts) and then the ratio cytosol/total extracts was calculated.

Lipids extraction and analysis.

Lipids were extracted by the method of Bligh and Dyer (Iverson et al., 2001) modified by us. Briefly, 25 eggs/embryos were homogenized in 1 ml of water using a Teflon potter. This homogenate was transferred to a glass tube and 5 ml of a solution 2:1 of methanol/chloroform was added. The tube was incubated overnight at -20°C. After centrifugation (5 min at 2800 rpm, SS34 rotor, Sorvall Instruments), a 1:1 chloroform/water solution was added to the supernatant and then vortexed. Following a second centrifugation step, the organic phase was collected, washed twice with 2 M KCl, then twice with water and finally dried under nitrogen. Afterwards, lipid samples were resuspended in 50 µl of chloroform and analyzed by two-dimensional thin layer chromatography of organic phase on Silica Gel H layers (pore size 60 Å, *Sigma Chemical Company*, St Louis, USA) developed in chloroform/methanol/water (75:30:1.5, v/v) and chloroform/methanol/acetic

acid/water (80:9:12:2, v/v). Phospholipids were revealed by staining with a $CuSO_4/H_3PO_4$ solution (10% w/v $CuSO_4$, 8% w/v H_3PO_4) (William, 2003). PC and PE were identified by co-migrating lipid standards, quantified using ImagePro3 software (Media Cybernetics) and normalized to the number of eggs/embryos used.

Statistical analysis.

Results are expressed as mean ± SEM. Data were analyzed by parametric test, using the t-Student and ANOVA. The 0.05 level of probability was used as the criterion of significance.

mRNA expression analysis.

RNA was isolated from *Bufo arenarum* unfertilized eggs using the TRIzol Reagent (*Invitrogen*, Carlsbad, CA, USA) following the manufacturer's instructions. Purified RNA was incubated with RQ1 DNAse (*Promega Corporation*, Madison, WI, USA). Total RNA was retro-transcribed with SuperScript II enzyme (Invitrogen, Carlsbad, CA, USA) and 16-18 oligo(dT). PCR amplification was carried out using the following primers: CTT_{forw}: 5'-AGGTGGAAGAGAAGAGCATCG-3', CTT_{rev}: 5'-GGAAGTCTTGCCAGAGAAGG-3'. The amplification reaction consisted of 2 μl of the cDNA template, 1.25 U GoTaq DNA Polymerase (*Promega Corporation*, Madison, WI, USA), 1.5 mM $MgCl_2$ (total reaction volume = 50 μl). The reaction mix was subjected to 35 cycles of amplification (95°C for 1 min, 52°C for 30 sec, 72°C for 30 sec), followed a final extension step of 10 min at 72°C. Products were resolved in 1.2% (w/v) agarose gels stained with Gel Green, extracted from them and finally cloned into pGEM-T Easy Vector (*Promega Corporation*, Madison, WI, USA). Sequencing was performed by the University of Maine DNA Sequencing Facility. Sequence identity was confirmed using the basic local alignment search tool (BLAST) supported by the National Center for Biotechnology Information. Multiple sequence alignments were carried out using the CLUSTAL W (1.82) algorithm (Higgins et al., 1996).

Results

CCT expression during development

By performing RT-PCR using CCT specific primers and RNA obtained from *Bufo arenarum* unfertilized eggs, we detected a band of the expected size (233 bp) (Fig. 1A). The PCR band was excised, cloned and sequenced as described in Materials and Methods.

The sequence of the amplified fragment is shown in Fig. 1B. The analysis of this sequence fragment revealed a high identity with CCT mRNA sequences of several vertebrates (data not shown) and the aminoacidic sequence comparison from different species shows a high degree of identity (Fig. 1C). This high identity score can be explained considering that the amplified region encoded the CCT membrane binding domain (Cornell and Taneva, 2006; Kent, 1997). To analyze CCTα expression in *B. arenarum* eggs, we used an antiserum raised against the rat CCTα. Fig. 1D shows that the antiserum recognises a unique band in eggs cytosolic samples that co-migrated with the positive control (rat liver extract). There was a slight difference in mobility that could be due to differences in primary sequence or different post-translational modifications (e.g. phosphorylation) (Arnold et al., 1997). CCT is an amphitropic enzyme that binds to cellular membranes (Cornell and Northwood, 2000). We evaluated by SDS-PAGE and western blots the presence of the enzyme in total extracts, cytosol, and total membrane fraction, using the anti-CCT antibody (Fig. 1F). In all the tested samples a band immunoreactive to the antibody was found. It is important to note that cytosolic samples rendered cleaner blots than total membranes samples and so they were preferentially selected in the experiments dealing with the estimation of CCT activity using membrane association as an indicator (see below).

CCT expression during different developmental stages (unfertilized egg to neurula) was analyzed by western blot (Fig. 2A). The levels of CCT were quantified using actin as its corresponding loading control. CCT protein levels remain high and constant until morula stage, where they show a statistically significant decrease reaching a minimum at the stage of gastrula after which they start a slow recovery (Fig. 2B). The observed decrease in the gastrula stage followed by a slow recovery coincides with events characteristic of MBT like the beginning of zygotic genes transcription (Newport and Kirschner, 1982).

CCT activity can be regulated by membrane association; a soluble homodimer is an inactive reservoir that is activated after binding to the lipid bilayer (Vance and Vance, 2004). As membrane biosynthesis is essential during development and CCT protein levels decrease in the stage of morula/gastrula (Fig. 2), we hypothesized that CCT could be regulated by its translocation to the membrane in order to increase its activity for supplying PC adequately. To test this, we measured CCT levels in cytosol samples and in total extracts and calculated the cytosol to total CCT ratio in selected stages (egg, blastula,

gastrula and neurula). Cytosolic and total extracts samples of staged embryos (N=3) were run together to ease quantification (Fig. 3A). Fig. 3B shows the result of densitometry and calculations of the ratios (see Materials and Methods). We observed that in all the stages analysed, approximately 50% of CCT is cytosolic and there is no statistically significant difference among them. Considering that total CCT diminished in blastula, gastrula and early neurula (Fig. 2), and the ratio of cytosolic to total CCT protein does not change, we can assume that the membrane associated (active) form of CCT per embryo is lower in these stages compared to eggs and embryos in early cleavage. However, it should be remembered that, according to Alonso et al. (1982a), there is PC biosynthesis during the first stages of *Bufo arenarum* development which continues until at least late gastrula.

CK expression during development

Mouse embryos homozygous for an insertional mutation in *Chka* allele were only recovered at the blastocyst stage but not at embryonic day 7.5 (Wu et al., 2008), indicating that CKα, as well as CCTα, is crucial for the early development of mouse embryos. We assayed CK protein abundance in total extracts from *Bufo arenarum* egg, blastula, gastrula and neurula stages by western blot (Fig. 4A) and the correspondent densitometry analysis is shown in fig. 4B. The results demonstrate that CK expression pattern in development is quite analogous to CCT behavior. Also, CK protein levels at neurula stage are similar to the gastrula levels probably indicating a slower recovery than the one observed for CCT.

PC and PE in eggs and embryos

Considering that PC and PE are the major phospholipids of cell membranes (Jackowski and Fagone, 2005), and since our results suggest that the regulatory enzymes of the Kennedy pathway (CCT and CK) undergo a decrease in their expression (protein abundance) along development, we investigated the content of PC, PE and calculated the PC/PE ratio per embryo. PC and PE were analyzed and estimated by bi-dimensional TLC and Copper/phosphoric stain (Marcucci et al., 2010) (Fig. 5A and B). In agreement with previous studies (Alonso et al., 1982a; Barassi and Bazan, 1974), our analysis demonstrated that the mass of PC and PE, and the ratio PC/PE is maintained in all the embryonic stages studied.

Discussion

Although several works have dealt with lipids composition and synthesis in amphibian eggs and embryos (Alonso et al., 1982a; Mes-Hartree and Armstrong, 1976, 1980; Petcoff et al., 2008), no studies have focused on the expression of the PC key biosynthetic enzymes during normal development in any vertebrate. We examined the expression of CCT and CK at the protein level in eggs/early embryos of *Bufo arenarum* and found that both enzymes are highly expressed at the very beginning of embryonic development (e.g. until 32 cells stage), after which they decrease along the morula, blastula and gastrula stages. Following this transient decrease, CCT and CK levels raise again at early neurula stage. This last behaviour could be explained by the fact that during MBT, zygotic gene transcription is activated and more mRNA is available for translation.

Data from different cell types endorse the view that when prompt PC synthesis is required, cells respond either by gene induction (transcription and translation) or CCT activity is up regulated based on its interaction with membrane lipid components (Jackowski, 1994; Sugimoto et al., 2008). Nevertheless, in the stages in which CCT expression decreases there is no indication of a compensatory increment of CCT activity by membrane association. Apparently, in our model none of these mechanisms seem to be activated, as the CCT protein level decreases and the cytosol/total extract ratio remains constant, though cell proliferation and membrane generation are undoubtedly going on.

At first glance, the decrease in CK and CCT content observed between the 32-cell stage to gastrula may seem unexpected in the context of membrane biogenesis requirements and the previous findings obtained from KO mice (Wang et al., 2005; Wu et al., 2008). *Bufo arenarum* is an amphibian that has external development. Externally developing embryos are limited to the resources contained within the egg, therefore, these embryos contain large reserves of lipid droplets, glycogen and yolk, which provide the energy and building blocks required for embryogenesis (Jorgensen et al., 2009). Also it is interesting to note that in other amphibians (*Rana pipiens*, *Triturus pyrrogaster*, *Cynops pyrrogaster* and *Xenopus laevis*) yolk consumption starts around the blastula - gastrula stages (Jorgensen et al., 2009; Karasaki, 1963; Komazaki et al., 2002), the same time that in *Bufo arenarum* CCT and CK enzyme levels are decreasing. The utilization of these reservoirs from which nascent

membranes may obtain their constituents could explain how cell division takes place even though key players of the Kennedy pathway (CCT and CK) show a considerable expression decrease during morula, blastula and gastrula stages. Further supporting this view, we also have examined PC and PE content per egg/embryo and there are no statistically significant differences among the evaluated stages. Similar findings regarding phospholipids content have been described in this species (*Bufo arenarum*) by other authors (Alonso et al., 1982a; Alonso et al., 1982b) and in *Xenopus laevis* (Mes-Hartree and Armstrong, 1976). The fact that PC and PE total content is unchanged along early development suggest that these phospholipids are in reservoirs large enough to supply the embryo initial needs. There are at least three mechanisms by which these phospholipids can be mobilized from the yolk platelets to the sites of membrane biogenesis: 1) diffusion through the cytoplasm; 2) vesicular transport; and 3) lipid transfer protein-mediated transport. Nevertheless, Rusiñol et al. (1987) suggested that *Bufo arenarum* fertilization does not activate protein-mediated phospholipid transfer.

As mentioned above, amphibian eggs not only have a reservoir of phospholipids but also a reservoir of maternal proteins and indeed we have shown that the amounts of CCT and CK proteins in the first stages analysed are high. In this connection, it is important to note that in a mammalian model these egg enzymes reservoirs also seemed to be high, as heterozygous mice for CK and CCT have no or very mild phenotype, further supporting the idea that enzyme levels are more than sufficient to maintain PC-associated functions (Wang et al., 2005; Wu et al., 2008).

In *Bufo arenarum* Alonso et al. (1982a) have shown that ^{32}P was actively incorporated into individual phospholipids in the absence of measurable net synthesis of phospholipids during the first stages of development (fertilized egg, late blastula, late gastrula). As phospholipids homeostasis is regulated by the expression and activity of biosynthetic enzymes and also by turnover through phospholipases activity (Jackowski, 1994), these results suggest that PC turnover plays an important role during early embryogenesis. Moreover, given that the net synthesis of PC in early development does not seem essential, it can be probably hypothesized that perhaps what matters in early development is not the end product of the pathway (e.g. PC) but the enzyme activities themselves, the intermediate metabolites of the route or the sub-cellular location where they are generated.

Altogether, previous published findings and the results presented in this manuscript suggest the existence in the embryo of mechanisms in charge to maintain the balance of phospholipids by regulating the activity of phospholipases that work in coordination with "*de novo*" synthesis of phospholipids and yolk platelets reservoir utilization to fulfil early developmental phospholipids needs.

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Conflict of Interest

None.

Figure legends

Figure 1 CCT expression by *B. arenarum* eggs. **(A)** Detection of CCT expression by RT-PCR. C-: negative control (skipped reverse transcription), cDNA: egg cDNA; C+: PCR performed using a plasmid harbouring rat CCT sequence. **(B)** Nucleotide sequence of the CCT fragment amplified using cDNA obtained from *B. arenarum* unfertilized eggs. **(C)** Aminoacidic sequence alignment of CCT from different species. Asterisks indicate aminoacid identity. **(D)** Increasing protein amounts of cytosolic samples from *B. arenarum* eegs were electrophoresed, blotted and revealed with anti-CCT antibody (3-12 µg of protein). C+: rat liver extract as positive control (10 µg of protein). The arrow points the band corresponding to CCT (42 kDa). **(E)** Coomassie Blue stained gel of total extracts (TE), cytosol (CYT) and total membranes (TM) obtained from eggs. Each lane was loaded with 10 µg of protein. Molecular standards are indicated to the right of the panel. **(F)** A similar gel (20 µg of protein) was blotted and revealed with anti-CCT antibody. The arrow indicates the band corresponding to CCT and the asterisks show nonspecific bands in the TM fraction.

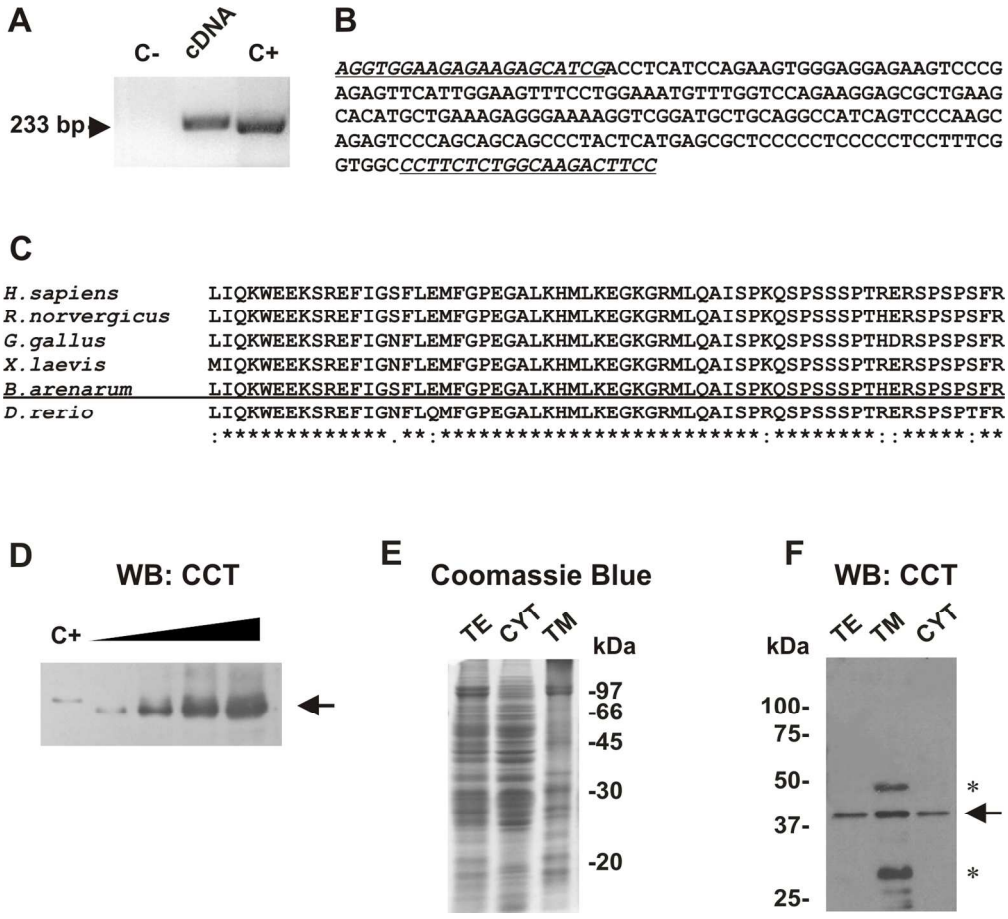
Figure 2 CCT protein expression during development. **(A)** Representative CCT western blot of total extracts from egg and embryos at different stages (10 µg protein per lane). Actin detection was performed to ensure equal loading. **(B)** Densitometric analysis of three independent experiments similar to the one in A.

Figure 3 Cytosolic vs. Total extract CCT protein in staged embryos. **(A)** CCT detected in egg and embryonic total extracts (TE) and cytosol (CYT) samples (from three independent fertilization events) by western blot (egg, blastula, gastrula and neural groove, 10 µg TE

protein and 15 μg CYT protein per lane). Actin detection was performed to ensure equal loading. **(B)** Densitometry analysis of the blot shown in A.

Figure 4 CK protein expression during development. **(A)** CK detected in egg and embryonic total extracts (from three independent fertilization events) by western blot (egg, blastula, gastrula and neural groove, 10 μg protein per lane). Actin detection was performed to ensure equal loading. **(B)** Densitometric analysis of the blot shown in A.

Figure 5 PC and PE in *Bufo arenarum* eggs and embryos. Total phospholipids were isolated from eggs and staged embryos (25 in each case) and analysed by bi-dimensional TLC. **(A)** Typical bi-dimensional TLCs revealed with Copper/Phosphoric reactive (one for each stage: egg, blastula, gastrula and neural groove). Close arrows indicate the point of loading of PC standard and open arrow of PE standard. **(B)** Semi-quantification of PC and PE in eggs and embryos (see Material and Methods for details)



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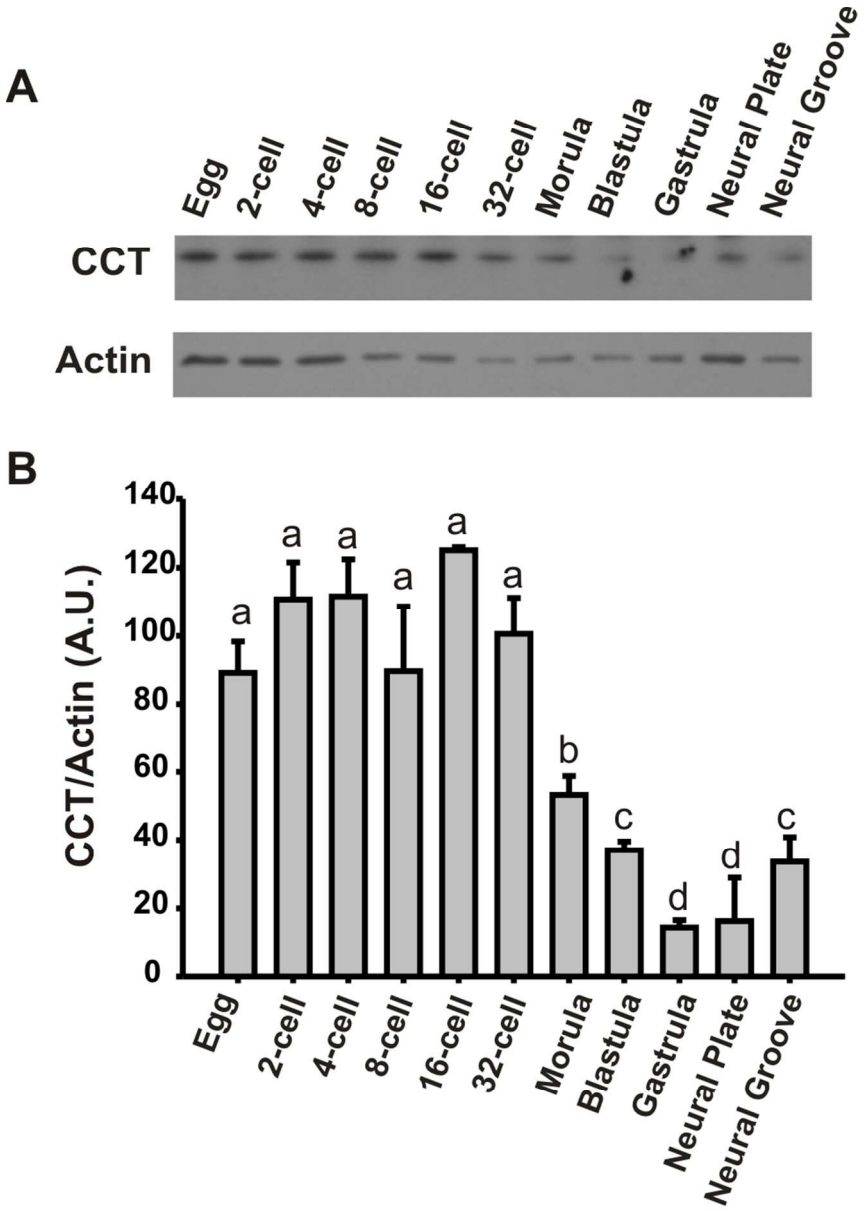

Figure 2 CCT protein expression during development. (A) Representative CCT western blot of total extracts from egg and embryos at different stages (10 µg protein per lane). Actin detection was performed to ensure equal loading. (B) Densitometric analysis of three independent experiments similar to the one in A.
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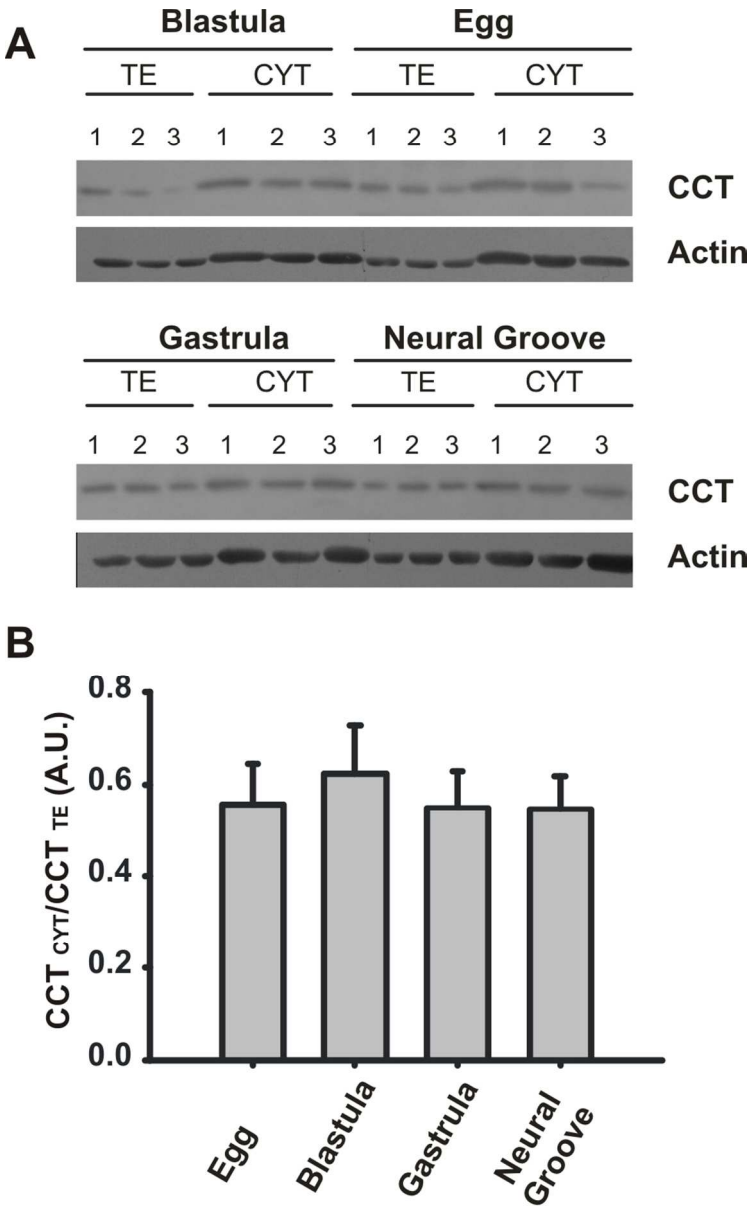

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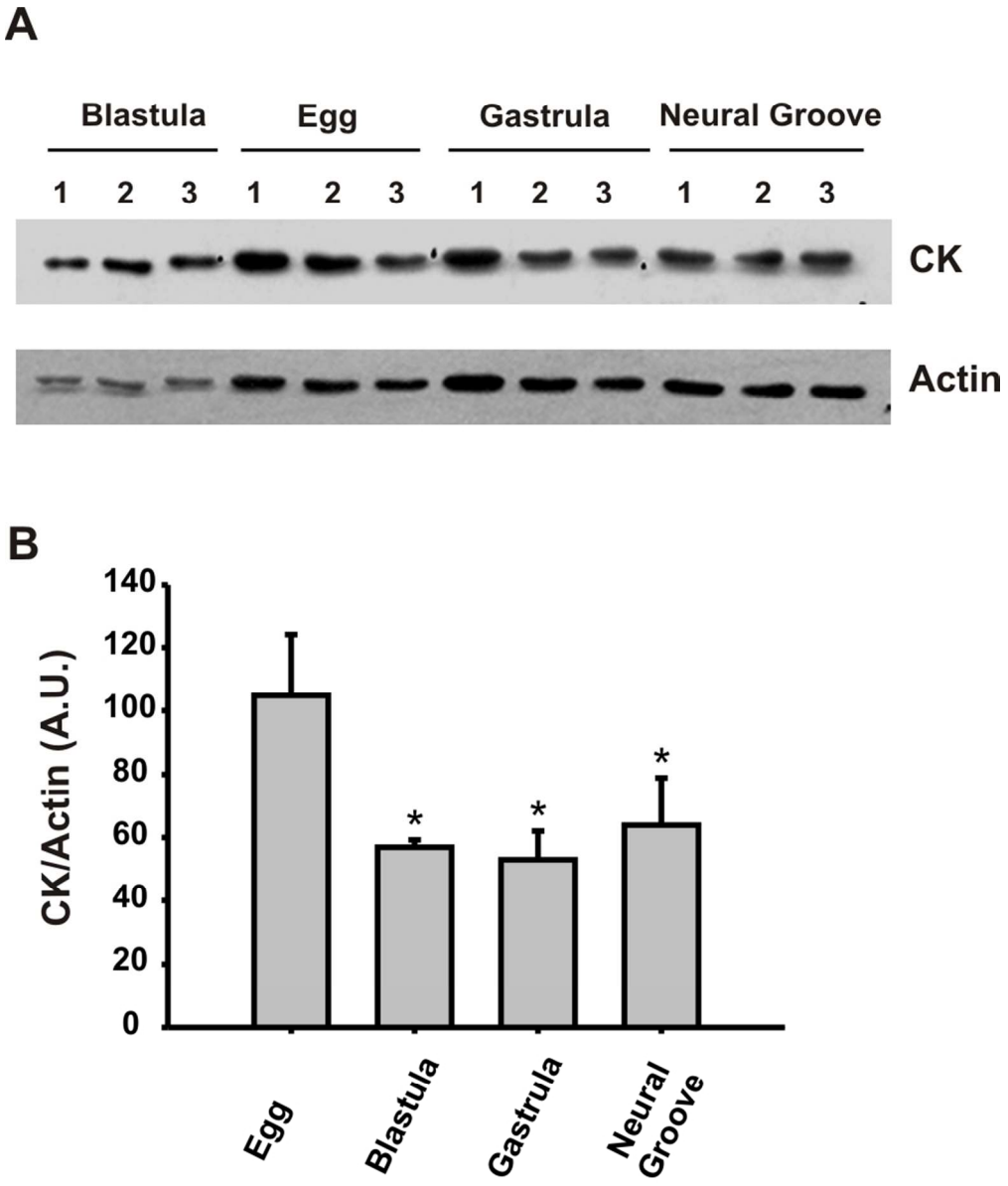

Figure 4 CK protein expression during development. (A) CK detected in egg and embryonic total extracts (from three independent fertilization events) by western blot (egg, blastula, gastrula and neural groove, 10 µg protein per lane). Actin detection was performed to ensure equal loading. (B) Densitometric analysis of the blot shown in A.
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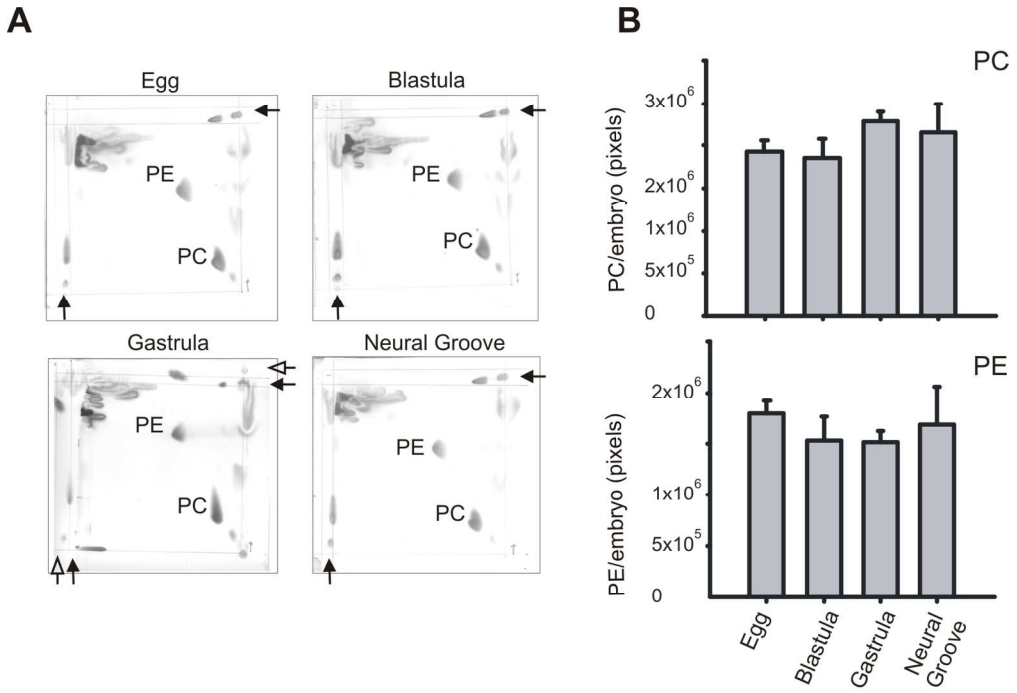

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148x101mm (300 x 300 DPI)