CALCIUM-BINDING PROTEIN OF THE CHICK CHORIOALLANTOIC MEMBRANE

II. Vitamin K-Dependent Expression

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ABSTRACT

A simple method was devised for the maintenance of the chorioallantoic membrane (CAM) of chick embryos in organ culture. Explants of CAM survived for up to 5 days in this system and retained the characteristic three-layered morphology (ectoderm, mesoderm, and endoderm). Induction of the CAM calcium-binding protein (CaBP) by effectors of calcium metabolism was studied in these organ cultures. Vitamin K was found to elicit a seven- to eightfold increase in CaBP, whereas no increase in CaBP activity occurred on supplementation with vitamin A, parathyroid hormone, an analogue of vitamin D, vitamin D and its hydroxylated metabolites, or with elevated calcium levels. The vitamin K-mediated induction of CaBP was dose-dependent, inhibited by the vitamin K antagonists warfarin and dicoumarol, selective for vitamin K_5, and maximal at the developmental stage (13–15 days of incubation) corresponding to the onset of calcium transport by the CAM in vivo. CaBP levels increased after 60–70 h in cultures of 13–15 day CAM supplemented with vitamin K and reached maximal levels around 80–90 h of culture. The CAM ectoderm underwent extensive proliferation and often assumed a villuslike morphology in the vitamin K cultures.

KEY WORDS calcium transport · calcium-binding protein · chorioallantoic membrane · embryonic development · vitamin K · organ culture

During skeletal calcification, the chorioallantoic membrane (CAM)[1] of the chick embryo is responsible for mobilizing the required calcium from the egg shell into the embryonic circulation (5, 12). A specific calcium-binding protein (CaBP) associated with the CAM has recently been identified (18) and purified (17, 19). Several properties of the CaBP suggest that it may be integrally involved in the calcium-transport function of the CAM. In particular, the CaBP is localized at the calcium-transporting cell layer, the ectoderm, of the CAM (20). Moreover, in vivo expression of the CaBP (18) is concomitant with the onset of calcium-transport activity in the CAM (16) and the accumulation of calcium by the embryo (11). Therefore, the presence of the CaBP in the CAM provides a useful biochemical marker for investi-

[1] *Abbreviations used in this paper*: anti-CaBP, antiserum prepared against purified CaBP (20); CaBP, calcium-binding protein; CAM, chorioallantoic membrane; Gla, γ-carboxyglutamic acid; NTC, basal culture medium with Neuman-Tytell Serumless Medium and 10% supplement of extracts prepared from 10-day-old chick embryos; TAD buffer, 13.7 mM Tris-HCl, pH 7.4, 0.12 M NaCl, 4.74 mM KCl, 98.5 μM glucose, 0.71 mM dithiothreitol, 0.02% sodium azide.

gating the mechanisms regulating the calcium-transport function of the CAM.

A most interesting molecular property of the CAM CaBP is that it contains several residues of a recently identified calcium-binding amino acid, γ-carboxyglutamic acid (Gla) (17, 19). Discovered originally in the blood-clotting factor, prothrombin (13), Gla has since been shown to be formed by a post-translational, vitamin K-dependent enzymatic reaction (14). The presence of Gla residues in the CAM CaBP therefore suggests that vitamin K-dependent mechanisms may be involved in the expression of the CaBP and in the regulation of calcium transport in the CAM. We report here the establishment of an in vitro organ culture system for the CAM to assess the possible functional role(s) of vitamin K and various known regulators of calcium metabolism in the expression of the CaBP in the CAM.

MATERIALS AND METHODS

Embryos

Fertilized white Leghorn eggs were purchased from Shamrock Farms (North Brunswick, N. J.) and incubated at 37.5°C in a humidified egg incubator for the desired periods of time. Eggs were routinely examined by transillumination for fertility and normal development of the embryo.

Materials

Neuman-Tytell Serumless Medium was purchased from Grand Island Biological Co. (Grand Island, N. Y.). The sources of the following substances were as indicated: penicillin, streptomycin, fungizone, and vitamin K_5 (4-amino-2-methyl-1-naphthol) (Grand Island Biological Co.); Bacto-Agar (Difco Laboratories, Detroit, Mich.); vitamin A (retinol palmitate), parathyroid hormone, dihydrotachysterol, and menadione sodium bisulfite (Sigma Chemical Co., St. Louis, Mo.); vitamin D_3 (cholecalciferol) (Aldrich Chemical Co., Inc., Milwaukee, Wis.); hyaluronidase (bovine), and Pronase (Calbiochem, San Diego, Calif.); Gill's hematoxylin and eosin Y (Polysciences, Inc., Warrington, Pa.; and bovine serum albumin (Armour, Phoenix, Ariz.). The hydroxylated derivatives of vitamin D_3, 25-hydroxycholecalciferol and 1,25-dihydroxycholecalciferol, were a gift from Dr. Uskokovic (Hoffman-La Roche Inc., Nutley, N. J.). All other chemicals used were of reagent grade.

Preparation of CAM Extract

The CAM was dissected from the embryo as described previously (18). The CAM extract was prepared by homogenizing the CAM, either freshly dissected or after organ culture (see below), in a Waring blendor (Waring Products Div., Dynamics Corp of America, New Hartford, Conn.) for four 15-s intervals with 4 vol (vol/wt) of 13.7 mM Tris-HCl, pH 7.4, 0.12 M NaCl, 4.74 mM KCl, 98.5 μM glucose, 0.71 mM dithiothreitol, 0.02% sodium azide (TAD buffer). The soluble CAM extract is defined as the supernate obtained after centrifugation at 31,000 *g* for 30 min.

Assay for Calcium-Binding Activity

Calcium-binding activity was measured by the Chelex 100-ion exchange assay as described elsewhere (18). Units of calcium-binding activity were calculated as:

$$\frac{\text{radioactivity in supernate}}{\text{radioactivity retained by Chelex 100}},$$

and expressed as a percentage.

In Vitro Organ Culture of CAM

To dissect the CAM under sterile conditions, the eggs were first sterilized externally by application of 0.1% alcoholic iodine. The egg was then cracked in the middle and its contents were placed in a sterile Petri dish. The CAM was carefully dissected out with sterile instruments, rinsed twice in physiological saline, and immediately used for organ culture.

Explants of CAM were maintained in Neuman-Tytell Serumless Medium (10) supplemented with 10% embryo extract from 10-day-old chick embryos prepared as follows: Chick embryos were removed from the egg with blunt forceps, washed briefly with physiological saline, and placed (1:1, wt/vol) in Tyrode solution containing 0.14 M NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5.6 mM glucose, pH 7.6. The embryos were homogenized in a Waring blendor for 5 min. After the extract was allowed to stir for 1–2 h at room temperature followed by 2–3 h at 4°C, the large tissue clumps were removed by centrifugation at 4,300 *g* for 30 min. The supernate was then digested with hyaluronidase (20 μg/ml) for 1 h at 4°C. Removal of insoluble material was achieved by centrifugation at 20,000 *g* for 90 min. The extract was further cleared by serial filtration through Millipore filters (Millipore Corp., Bedford, Mass.) (8 μm, 3 μm, and 0.8 μm). The final embryo extract was sterilized by filtration (Millipore, 0.45 μm) and stored at −70°C until use.

The following formulation per 100 ml of basal culture medium with Neuman-Tytell Serumless Medium and 10% supplement of extracts prepared from 10-day-old chick embryos (NTC) was used: 90 ml of Neuman-Tytell Serumless Medium, 10 ml of chick embryo extract, 5,000 U of Penicillin, 5,000 μg of streptomycin, and 12.5 μg of fungizone. Freshly dissected whole CAM were placed in 100 × 10 mm Petri dishes containing 7 ml of the medium each, and the CAM was flattened onto the bottom of the dish to allow maximum contact with the culture medium. The cultures were incubated at 37°C under 5% CO_2 in a humidifed incubator.

The CAM explants were removed from the culture medium after the desired period of incubation, washed

thoroughly with physiological saline, and immediately frozen at −70°C until use.

Light Microscopy

Tissues were prepared for light microscopy by standard histological techniques (6). Samples were embedded in paraffin and sectioned at 4–6 μm thickness. The sections were routinely stained with Gill's hematoxylin (4) and counter stained with eosin.

Protein Determination

Protein was estimated by the method of Lowry et al. (7) with bovine serum albumin as a standard.

Immunological Methods

The preparation of the antiserum to CaBP (anti-CaBP), Ouchterlony-immunodiffusion, and immunohistochemistry were performed as described in the preceding report (20).

RESULTS

In Vitro Organ Culture of CAM

The CAM, due to its embryonic nature, is readily amenable to in vitro cultivation, and had been maintained on grids in Trowell-type organ culture dishes (3) and on agar surfaces (9). In the present study, an improved semi-suspension culture technique was developed using a liquid medium (see Materials and Methods). Compared to previous techniques (3, 9), the present procedure required relatively simple handling of the CAM, and large-scale culture could be efficiently maintained.

The integrity of the CAM explants maintained in this organ culture system for various periods of time was assessed by light microscope observations, and compared with that before culture (Fig. 1*A*). After 2 days in culture (Fig. 1*B*), the CAM explant appeared to be largely devascularized and, occasionally, regions of cellular proliferation were present in the mesoderm (Fig. 1*B*). After 3–4 days in culture (Fig. 1*C*), the ectoderm began to proliferate such that the original columnar epithelium assumed a stratified appearance with multiple layers of cells. The squamous endodermal epithelium, on the other hand, underwent little morphological change even up to 5 days in culture. Some cell death was observed in the mesoderm as indicated by the presence of pycnotic nuclei. This was probably due to the limited diffusion of nutrients in the culture medium into the centrally located mesoderm of the CAM. At the periphery of the explants, the ectoderm and the endoderm sometimes fused to form a continuous sheat surrounding the mesoderm. In general, the CAM explants appeared to survive adequately throughout the entire culture period (4–5 days) used in the present study, and their three-layered architecture (ectoderm, mesoderm, and endoderm) remained relatively intact.

Calcium-Binding Activity Expressed by the CAM during Culture

The basal culture medium (NTC) contained a 10% supplement of extracts prepared from 10-day-old chick embryos. Since the calcium transport function (16) and the calcium-binding activity (18) of the CAM are not expressed in 10-day-old embryos, potential effectors of these functions of the CAM should be absent in these embryo extracts. This was tested by measuring the calcium-binding activity levels in CAM explants as a function of culture time. Due to the developmental dependence of the calcium-binding activity in the CAM (18), the membranes were explanted for organ culture from embryos of four arbitrary age groups designated according to the levels of CaBP (18). These were: (*a*) 10–12 days of incubation, before the onset of CaBP expression; (*b*) 13–15 days, at the onset of CaBP expression; (*c*) 16–18 days, during rapid CaBP expression; and (*d*) 19–20 days, at maximal CaBP levels. The results are shown in Fig. 2. In each of the age groups studied, the specific activity (activity/mg protein) of calcium binding in extracts of the cultured CAM remained unchanged up to 4–5 days in culture. In addition, the amount of protein and the level of calcium-binding activity per unit wet weight of CAM explant were also relatively constant throughout the culture period.

These results therefore indicate that the *in vitro* culture system could be employed to study the effects of exogenously added factors on the calcium-binding activity of the CAM explants.

Effect of Exogenous Vitamin K and Other Factors on the Calcium-Binding Activity of CAM Explants

From the previously established age profile of calcium-binding activity in the CAM during embryonic development (18), it was reasonable to assume that at the onset of increased calcium binding and transport (around incubation day 13–

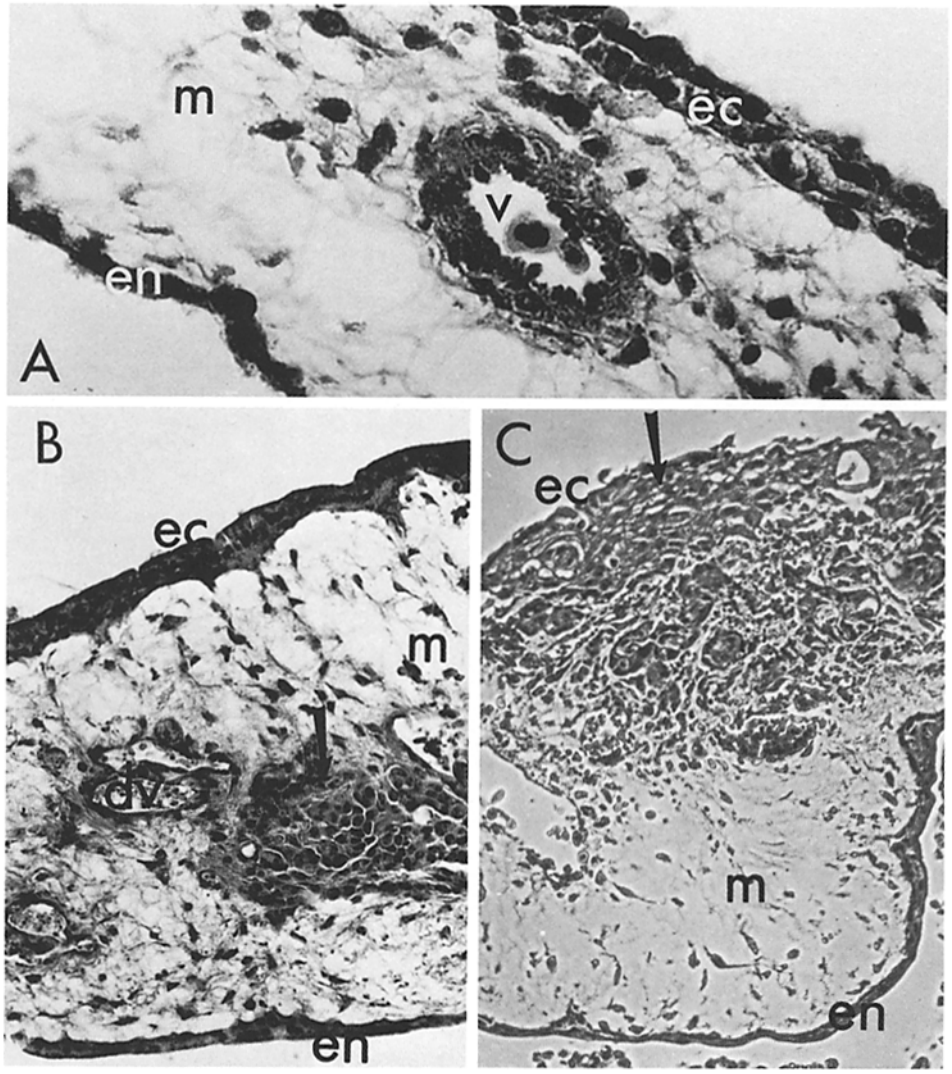

FIGURE 1 Morphology of 13- to 15-day-old CAM before and after *in vitro* organ culture. All paraffin sections of the CAM were 4–6 μm thick and were stained with Gill's hematoxylin (4) and eosin according to standard procedures (6). (*A*) 13-day-old CAM before culture showing the three-layered morphology (ectoderm, *ec*; mesoderm, *m*; and endoderm, *en*). A large blood vessel (*v*) was also present in the mesoderm. × 600. (*B*) 15-day-old CAM cultured in NTC medium for 2 days. The three-layered morphology (*ec*, *m*, and *en*) remained but signs of devascularization (*dv*) were apparent. Regions of cellular proliferation (arrow) also appeared in the mesoderm. × 280. (*C*) 14-day-old CAM cultured in NTC medium for 3½ days. Although the three-layered morphology was retained, there was a considerable proliferation of the ectoderm (arrow). × 400.

15), the expression of the CaBP in the CAM was necessarily under physiological regulation and hence should be most sensitive to potential effectors in the in vitro culture medium. Therefore, CAM explants were obtained from 13- to 15-day-old embryos and used in all experiments to test for the in vitro effects of various exogenous factors on the calcium-binding activity in the CAM.

The results of these experiments are shown in Fig. 3. The calcium-binding activity presented here is the difference between the specific activity (activity/mg protein) of CAM extracts after var-

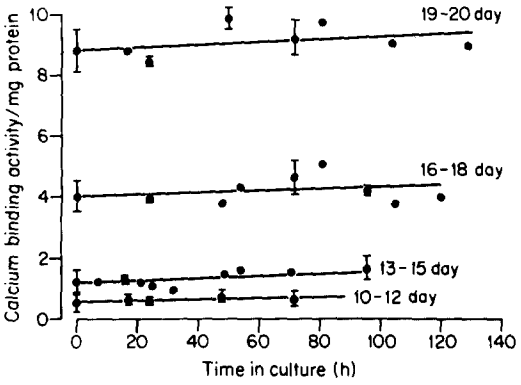

FIGURE 2 Effect of in vitro culture on the calcium-binding activity in CAM explants. The explants of CAM were obtained from chick embryos of the indicated ages and were cultured in NTC medium as described in Materials and Methods for various periods of time. Extracts were prepared from the CAM explants as described in Materials and Methods and were assayed for calcium-binding activity by the Chelex 100 method (18). The specific calcium-binding activities (activity/mg protein) are the means ±SEM of three to five separate determinations on extracts of four pooled CAM each.

ious times in culture and that before culture. This was a valid normalization since there was no significant change in the specific activity of calcium-binding in the CAM throughout the duration of in vitro culture in normal NTC medium (Fig. 2).

As shown in Fig. 3 A, the addition of vitamin A, vitamin D_3, and its hydroxylated metabolites, parathyroid hormone, and dihydrotachysterol in the culture medium produced no statistically significant changes in the level of specific calcium-binding activity in the CAM explants. Similarly, only a slight increase in activity was obtained for CAM explants cultured in the presence of elevated levels of $CaCl_2$.

Fig. 3 B shows the effect of vitamin K supplementation on the calcium-binding activity in the CAM cultures. A marked enhancement of the calcium-binding activity was apparent around 60–70 h in culture and reached a maximal level of more than 7- to 8-fold over that of the controls (see Fig. 2). It was found that cell-free extracts of CAM incubated with vitamin K and subsequently extensively dialyzed to remove vitamin K showed no similar enhancement in calcium-binding activity. Moreover, Pronase treatment (1:10, enzyme:CAM protein) at 37°C for 15 min (18) of extracts prepared from vitamin K-supplemented CAM explants abolished their calcium-binding activity. These findings therefore indicate that the inductive effect of vitamin K is cell-dependent and results from a proteinaceous substance(s).

Our previous results had indicated that the enhanced calcium-binding activity of calcium-transporting CAM is due to a single species of CaBP (18). That the vitamin K-induced calcium-binding activity in CAM explants resulted from the expression of this CaBP was demonstrated by immunological methods. Fig. 4 shows the Ouchterlony double immunodiffusion patterns of cultured CAM extracts with anti-CaBP antiserum. The increased intensity of the precipitin line against the extract of CAM cultured in vitamin K-supplemented medium compared to that against controls indicates that the absolute amount of CaBP increased in the vitamin K-containing cultures. Comparison of the anti-CaBP immunofluorescent staining patterns of vitamin K-treated

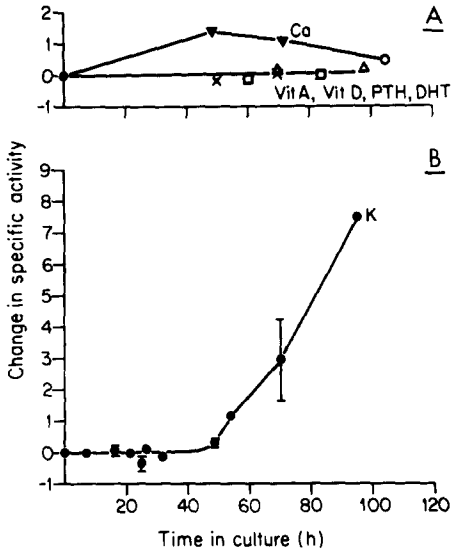

FIGURE 3 Effect of exogenous factors on the calcium-binding activity in organ-cultured CAM. The CAM explants were obtained from 13- to 15-day-old embryos and were cultured and assayed for calcium-binding activity as described under Fig. 2. The exogenously added substances were: A, *Ca*, $CaCl_2$ (5 or 10 μmol/ml); *Vit A*, vitamin A (10 or 20 μg/ml); *Vit D*, vitamin D_3 (2, 5, or 10 μg/ml), 25-hydroxy vitamin D_3 (25 μg/ml), or 1,25-dihydroxy vitamin D_3 (0.25 μg/ml); *DHT*, dihydrotachysterol (100 μg/ml); *PTH*, parathyroid hormone (2 or 4 U/ml); and B, *K*, vitamin K_5 (10 μg/ml). The change in specific activity (activity/mg protein) represents the difference between levels of specific calcium-binding activity in extracts of CAM before and after culture for the indicated periods.

and control CAM explants demonstrated that the former stained more intensely and therefore contained more CaBP. The vitamin K-induced CaBP staining in the CAM explants appeared gradually as a function of culture time and was most evident after 2–3 days in culture, indicating a qualitative correlation with the increase in the specific calcium-binding activity (Fig. 3 B). Furthermore, the CaBP staining in the vitamin K-treated CAM explants appeared to be located primarily on the ectoderm and frequently in the form of clusters. Morphologically, the general integrity of the three-layered CAM (ectoderm, mesoderm, and endoderm) was preserved in the vitamin K-supplemented cultures (Fig. 5) as well as the control cultures (Fig. 1*A*). However, the ectoderm usually underwent extensive proliferation in vitamin K-supplemented cultures to form highly stratified columnar epithelia which sometimes assumed a villuslike appearance.

Characterization of the Vitamin K Induction of CaBP Expression by the CAM

DEPENDENCE ON TIME OF ADMINISTRATION: The time lag (60–70 h) in the response of organ-cultured CAM to vitamin K prompted a study of whether a continuous presence of vitamin K in the culture medium was necessary to elicit the induction. Explants of CAM were obtained from 13- to 15-day-old embryos and cultured in NTC medium with or without vitamin K supplement for 48 h. They were then removed, washed in physiological saline, and transferred to normal and vitamin K-supplemented NTC media, respectively. The results shown in Fig. 6 demonstrate that the presence of vitamin K in the first 48 h of culture was essential and probably sufficient for the subsequent induction of CaBP expression. On the other hand, vitamin K supplementation after 48 h of culture in normal NTC medium was ineffective in eliciting this inductive response. The minimal time required to elicit the inductive response was not studied.

DOSE DEPENDENCE: The results in Table I indicate that the inductive effect of vitamin K was dose-dependent and that the optimal dose was 5–10 μg/ml (29–58 μM). Another structural and functional homolog of vitamin K, menadione (vitamin K_3), was also tested for CaBP inductive activity (Table I). With 10 μg/ml (30 μM) of menadione in the culture medium, the induction

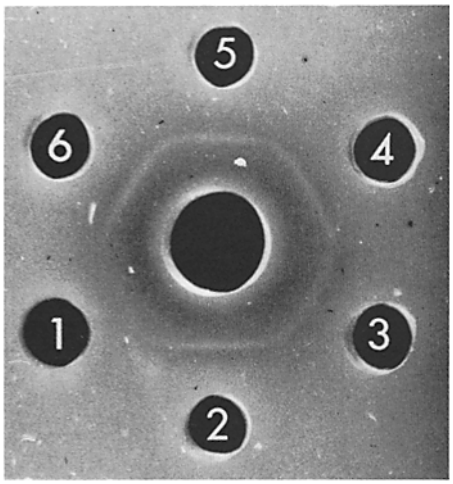

FIGURE 4 Ouchterlony double immunodiffusion of extracts of CAM explants against anti-CaBP. The extracts (5 mg protein/ml) were prepared from CAM explants cultured for 3 days in the following media: *1*, normal NTC medium; *2–6*, vitamin K-supplemented medium (10 μg/ml) from separate experiments. 10 μl of each extract was placed in the designated well, and the center well contained 10 μl of anti-CaBP. Diffusion was allowed to proceed for at least 48 h at room temperature.

of calcium-binding activity in CAM explants was 50% of that with vitamin K_5 at a comparable concentration.

INHIBITION BY VITAMIN K ANTAGONISTS: The vitamin K specificity of the in vitro induction of CaBP expression was further demonstrated by the incorporation of vitamin K antagonists, such as warfarin and dicoumarol, at 50 μg/ml into the culture medium. Their effects on the expression of calcium-binding activity in the CAM explants are shown in Fig. 7. Warfarin (or dicoumarol) suppressed the vitamin K-dependent induction of calcium-binding activity in the CAM explants. The activity levels of these cultures approximated that of controls cultured in NTC medium. Moreover, in cases where both vitamin K and warfarin were included in the culture medium, the CaBP-inductive response was substantially delayed and reduced. At the concentration of warfarin (or dicoumarol) used, the CAM appeared viable from morphological observations. However, proliferation of the ectoderm characteristic of vitamin K-supplemented cultures (see Fig. 6) was not observed.

DEPENDENCE ON THE DEVELOPMENTAL AGE OF THE CAM EXPLANT: As shown in Table II, non-calcium-transporting CAM (10–12

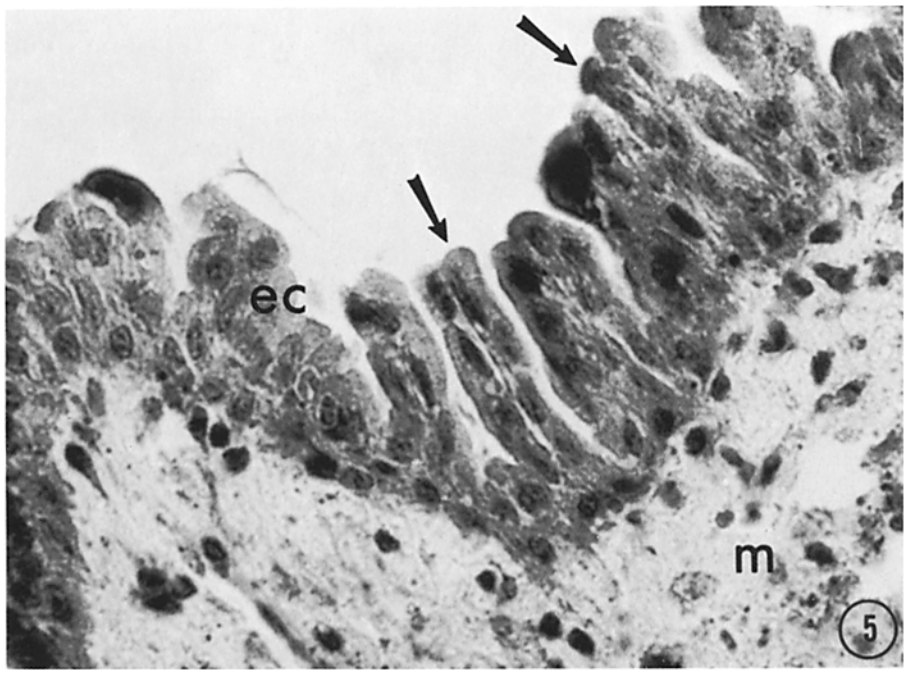

FIGURE 5 Morphology of 15-day-old CAM cultured for 3 days in NTC medium supplemented with vitamin K (10 μg/ml). The ectoderm (*ec*) proliferated extensively and gave rise to villuslike structures (arrows). *m*, mesoderm. × 730.

day) were not responsive to vitamin K induction. For calcium-transporting CAM, the highest induction was observed with 13–15 day CAM, whereas CAM obtained from mid- (16–18 day) and late- (19–20 day) stages of calcium transport showed relatively less and delayed responses to vitamin K. These results indicate that the vitamin K-dependent induction of calcium-binding activity in the in vitro CAM cultures was highest at 13–15 days of development, and coincident with the onset of calcium transport (16) and CaBP expression (18) by the CAM.

DISCUSSION

In this investigation, we have developed a simple procedure for maintaining chick CAM explants in organ culture. We have employed this in vitro system to study the effects of various known effectors of calcium metabolism on the expression of the CaBP by the CAM. Our results show that vitamin K induces the expression of CaBP, whereas vitamins A and D, parathyroid hormone, and elevated levels of calcium are ineffective. This report, therefore, presents, for the first time, evidence for a specific and unique role of vitamin K in the regulation of a calcium transport-associated function.

Further supporting evidence for the vitamin K specificity of CaBP induction in the CAM include: dose-dependence, selectivity for a specific vitamin K analogue, and inhibition by vitamin K antagonists. These features of the vitamin K-mediated induction of CaBP in the CAM are reminiscent of the vitamin K-dependent production of several blood coagulation proteins, such as prothrombin (15). Administration of vitamin K to vitamin K-deficient animals or to liver tissues isolated from these animals also elicits the appearance of prothrombin in a dose-dependent manner which is inhibited by coumarin (15). It is likely therefore that in vivo vitamin K-dependent mechanisms may regulate the expression of the CAM CaBP. In support of this idea, our recent studies (to be published) indicated that injection of the vitamin K antagonist warfarin into the developing chick embryo inhibited the expression of the CaBP in the CAM.

The egg yolk is one of the richest natural reservoirs of vitamin K, containing ~40–50 μg of the vitamin (1). Although little is known concerning the mobilization of vitamin K in the chick embryo, it is conceptually possible that the gradual release of vitamin K from the egg yolk into the developing CAM ectoderm constitutes the control

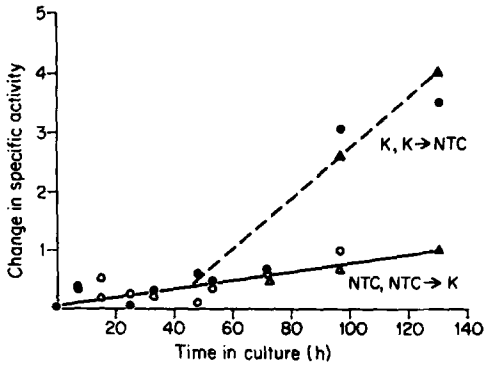

FIGURE 6 Relationship between expression of calcium-binding activity in CAM explants and the time of vitamin K administration. Explants of CAM obtained from 13- to 15-day-old embryos were cultured as described in Materials and Methods in the following media: (*a*) ○, in NTC medium alone; (*b*) △, for 48 h in NTC and then transferred to vitamin K-supplemented NTC (*K*); (*C*) ●, in vitamin K-supplemented NTC; and (*d*) ▲, in vitamin K-supplemented NTC for 48 h and then transferred to NTC. Extracts of the CAM explants were prepared and assayed as described under Fig. 2.

TABLE I

In Vitro Vitamin K Induction of Calcium-Binding Activity in the CAM*

Dose dependence	
Concn	Response‡
μg/ml	%
1	19 ± 2
2	52 ± 6
5	100 ± 10
10	87 ± 10
10 (K_3)§	50 ± 3
20	76 ± 12

* Vitamin K_5 was used in all cases except as indicated.
‡ Calcium-binding activities at maximal induction expressed as a percentage of that at 5 μg vitamin K_5/ml.
§ Vitamin K_3 was used in the form of the water-soluble menadione sodium bisulfite.

mechanism governing the characteristic age-dependent expression of the CaBP (18). The gradual increase in vitamin K levels in the CAM may be a simple consequence of the increased vascularization of the CAM during embryonic development (2), thereby providing a continuous source of the vitamin to the surrounding cells.

The molecular role of vitamin K in the expression of prothrombin is known (14). A vitamin K-dependent post-translational modification of prothrombin results in the γ-carboxylation of 10–14 glutamate residues (13). These Gla residues on the prothrombin molecule are responsible for the calcium-binding properties of the protein which are necessary for its subsequent activation to thrombin (15). However, the relatively small number (2–10) of Gla residues present in the CaBP does not appear to be sufficient to account for its high calcium-binding affinity and large number of calcium-binding sites (17, 19). This suggests that the vitamin K-dependent γ-carboxylation reaction may not be directly involved in the generation of calcium-binding activity of the CAM CaBP. The expression of CaBP may, therefore, be regulated at a nontranslational level by other vitamin K-mediated mechanisms.

The time lag (~3 days) between exposure of CAM explants to vitamin K and the onset of CaBP induction (Fig. 3 B) further supports the notion that vitamin K may be functioning in the CAM at a nontranslational level. In comparison, induction of prothrombin requires <60 min after the initial in vivo or in vitro administration of vitamin K (15). Because of these differences, it seems likely that the vitamin K-dependent processes regulating the expression of CaBP in the CAM explants may be mediated by additional cellular functions acquired at later developmental stages, and may, therefore, depend on the differentiated state of the CAM explant used for culture. This supposition is, indeed, consistent with our observation that the highest response to in vitro vitamin K induction was obtained with CAM explants of a specific developmental stage, 13–15

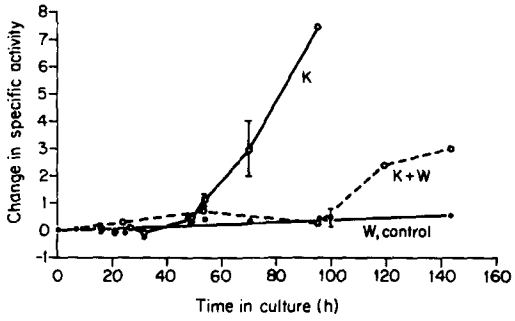

FIGURE 7 Effects of vitamin K and warfarin on the in vitro expression of calcium-binding activity in CAM. The explants were cultured in the following media: *control*, NTC medium; *K*, NTC medium supplemented with vitamin K (10 μg/ml); *W*, NTC medium with warfarin (or dicoumarol) (50 μg/ml); *K* + *W*, NTC medium with both vitamin K and warfarin (or dicoumarol) at the above concentrations. The CAM explants were cultured and assayed as described under Fig. 2.

TABLE II

Age-Dependent Inductive Effect of Vitamin K on the In Vitro Expression of Calcium-Binding Activity‡ in the CAM*

Age of CAM	sp act before Culture	sp act at Time of maximal induction	% Maximal induction§	Time of maximal induction
days				*h*
10–12	0.7 ± 0.1	0.7 ± 0.2	0	–
13–15	1.2 ± 0.2	8.4 ± 0.4	600	60–100
16–18	4.5 ± 0.5	6.7 ± 1.4	40	70–90
19–20	9.0 ± 0.8	13.8 ± 0.8	60	100–120

* Concentration of vitamin K_5 = 10 μg/ml.

‡ The specific calcium-binding activities (activity/mg protein) are the means ± SEM of three to five separate experiments on extracts of four pooled CAM each.

§ This is calculated from the specific calcium-binding activity (activity/mg protein) in CAM extracts prepared from explants before and after in vitro culture as:

$$\frac{\text{maximal sp act during culture} - \text{sp act before culture}}{\text{sp act before culture}} \times 100\%.$$

days of incubation (Table II).

Our morphological studies provide further indirect evidence for this supposition. These observations showed that the ectoderm of CAM explants appeared to proliferate after 2–3 days in culture and that the original columnar epithelium of the ectoderm became a stratified structure (Fig. 1 *C*). In the presence of vitamin K, the ectoderm of the CAM explants proliferated more extensively than controls and often gave rise to a villuslike morphology (Fig. 5). These morphological changes in the CAM ectoderm during in vitro cultivation are significant in light of the concomitant increased level of CaBP in the vitamin K-supplemented CAM explants. Several interesting possibilities exist. First, vitamin K may induce the proliferation of specific ectodermal cells which are responsible for the production of CaBP, resulting, therefore, in a net increase of the level of CaBP in vitamin K-supplemented CAM explants. Secondly, it seems possible that the ectodermal proliferation of the vitamin K-supplemented CAM explants may be accompanied by cellular differentiation, particularly since the final villuslike morphology differs significantly from the original columnar epithelium. If so, vitamin K may be responsible for inducing the differentiation of certain ectodermal cells into CaBP-producing cells which would also result in a net increase of CaBP in these CAM explants.

A recent study on the production of prothrombin in cultured hepatoma cells (8) suggested that vitamin K may function at a transcriptional level. For the CAM system, further studies employing techniques to monitor the synthesis of protein, RNA, and DNA and the post-translational reactions are certainly needed to elucidate the mechanism regulating the expression of the CaBP.

Until recently, vitamin K was thought to be primarily involved in the production of clotting factors (14, 15). The recent findings of Gla residues in various tissues concerned with calcium metabolism (see references in reference 14), however, have prompted suggestions that vitamin K may be an important humoral effector of calcium metabolism, in addition to the three known factors, parathyroid hormone, vitamin D, and calcitonin. The findings reported here strongly indicate that, during embryonic development of the chick, vitamin K may be integrally involved in calcium metabolism by regulating the functional state of the calcium-transporting CAM. Further investigations at the cellular and molecular levels are certainly necessary to elucidate this important regulatory aspect of calcium metabolism.

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