Supplemented eggshell restores calcium transport in chorioallantoic membrane of cultured shell-less chick embryos

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SUMMARY

It was previously reported (Tuan, 1980a) that the development-specific expression of calcium transport and related functions in the chick embryonic chorioallantoic membrane (CAM) requires the continuous presence of the eggshell, the calcium source of the embryo. To further understand the mechanism of action of the eggshell on the CAM functions, this study reports the effects of eggshell supplementation on chick embryos maintained in shell-less cultures. The cultured embryos were able to accumulate and utilize the exogenous shell calcium, applied directly onto the CAM, for skeletal formation. In the region of the CAM directly adhering to the added shell, calcium transport activity, calcium-binding protein (CaBP) activity, and vitamin K-dependent γ-glutamyl carboxylase activity were significantly restored. These results strongly suggest that the proximity of shell calcium may regulate expression of calcium transport and related functions in the chick embryonic CAM.

INTRODUCTION

During embryonic development of the chick, calcium is supplied to the embryo from two sources: the egg yolk and the eggshell (Simkiss, 1961; Johnston & Comar, 1955). Up to approximately the 9–10th day of incubation, the yolk appears to be the sole supplier of calcium. After this period, on the 11–12th day, shell calcium begins to be mobilized and calcium content of the embryo increases rapidly during the rest of incubation (Romanoff, 1967). Overall, the eggshell is the principal source of calcium for the chick embryo and contributes over 80% of the total calcium in the hatching chick.

The organ/tissue responsible for translocating shell calcium into the chick embryo is the extraembryonic chorioallantoic membrane (CAM) which lines the internal surface of the shell membrane and the eggshell (Terepka, Coleman, Armbrrecht & Gunther, 1976). Calcium transport by the CAM is a highly developmentally regulated function; activity begins around incubation day 12–13, rapidly increases in level thereafter, and reaches a maximal level around day 19–20 (Terepka, Stewart & Merkel, 1969; Tuan & Zrike, 1978). Our recent

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study (Tuan, 1980a) showed that the development-specific expression of calcium transport activity in the CAM requires the presence of the substrate itself, the eggshell. Chick embryos which develop in long-term shell-less cultures in vitro fail to exhibit the characteristic age-specific onset of calcium transport in their CAM. Biochemical analysis (Tuan, 1980a) revealed that the CAM calcium-binding protein (CaBP), a protein previously shown to be involved in the calcium transport function (Tuan & Scott, 1977; Tuan, Scott & Cohn, 1978a, b) is expressed as an inactive form (and in increased amount) in the CAM of these embryos. These findings strongly suggest that the eggshell may regulate CAM calcium transport at a cellular and biochemical level. This notion is particularly intriguing since direct attachment of the CAM to the shell membrane and eggshell takes place around incubation day 9–10 in vivo, i.e. shortly before the onset of calcium transport.

The goal of the present study is to directly assess the nature in which the proximity and/or availability of eggshell calcium influence the calcium transport function of the CAM. The approach taken here is to exploit the shell-less cultured chick embryos (1) to directly supplement eggshell to these embryos and (2) to characterize the status of calcium transport and related functions in the shell-supplemented (SS) embryos in comparison to normal (N) (i.e. in ovo) and shell-less (SL) embryos. The results reported here indicate the efficacy of experimental shell calcium supplementation to the cultured embryos and furthermore provide evidence that the expression of calcium transport activity and of functional CaBP in the CAM are strictly dependent on its direct proximity to eggshell calcium.

MATERIALS AND METHODS

Chick embryos

Fertilized chicken eggs were obtained from Shaw Poultry (West Chester, PA) and were incubated at 37.5 °C in a humidified commercial egg incubator for the desired period of time. Chick embryos were placed into shell-less culture in vitro after 3 days of incubation in ovo using a previously described procedure (Dunn & Boone, 1976; Tuan, 1980a; Dunn, Fitzharris & Barnett, 1981). The cultured embryos were incubated at 37.5 °C in a humidified tissue culture incubator with continuous air flow. For shell calcium supplementation, eggshell and adhering shell membranes were obtained from unincubated eggs, autoclave-sterilized, and were applied (two to three pieces of 5–6 cm² per embryo) with the original internal surface facing downward on top of the CAM of the cultured embryos on incubation day 11 (i.e. after 8 days in shell-less culture). To trace the tissue distribution of the supplemented calcium, eggshell was radiolabelled by soaking for three weeks in water containing ⁴⁵CaCl₂ (0.6 μCi/ml, New England Nuclear, Boston). The shell was then repeatedly rinsed with water to remove unincorporated ⁴⁵Ca and used for shell calcium supplementation as described above.
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After various periods of culture, the accumulation of shell calcium in several embryonic tissues was determined by liquid scintillation counting of \textsuperscript{45}Ca radioactivity in ashed (650 °C, 48 h) and acid-solubilized tissues.

Overall growth and development of the chick embryos was assessed by Hamburger-Hamilton staging (Hamilton, 1952). Comparison of embryonic skeletal formation was based on the weight, overall size, and size of the calcified regions of the following bones: calvaria and mandibles (both membrane bones) and femurs and tibia (both endochondral long bones).

CAM

Calcium uptake by the CAM was measured as described previously (Crooks & Simkiss, 1975; Crooks, Kyriakides & Simkiss, 1976; Tuan & Zrike, 1978). Briefly, buffer containing 1 mM CaCl\textsubscript{2} and a tracer amount of \textsuperscript{45}Ca(1–3 \textmu Ci) was introduced into a transport chamber constructed on top of the CAM. After 15 min of incubation, the buffer was removed and the radioactivity retained by the segment of the CAM underlining the chamber was determined. Calcium uptake activities were expressed as nmole calcium/min/cm\textsuperscript{2}.

Extracts of CAM were assayed for the CaBP by calcium-binding activity measurements and immunochemistry. The Chelex 100 (Bio-Rad) ion-exchange method (Tuan & Scott, 1977) was used to determine calcium-binding activity (units/mg protein). Single radial immunodiffusion using specific rabbit-derived anti-CaBP antibodies was employed for the immunochemical quantitation of the CaBP (\mu g/mg total protein) (Tuan, 1980a).

Vitamin K-dependent \gamma-glutamyl carboxylase activity in the CAM was assayed by measurement of vitamin K-dependent incorporation of \textsuperscript{14}CO\textsubscript{2} into endogenous microsomal proteins of the CAM using a previously published procedure (Tuan, 1979). Briefly, microsomes of the CAM were prepared by differential centrifugation and incubated in the assay mixture containing vitamin K\textsubscript{1} (100 \mu g/ml, Merck, Sharp & Dohme) and NaH\textsuperscript{14}CO\textsubscript{3} (0.1 mCi/ml, New England Nuclear) at 37 °C for 1 h. After removal of unincorporated \textsuperscript{14}CO\textsubscript{2} by acidification and dialysis, the protein-bound radioactivity in the samples (c.p.m./mg dry weight) was determined by liquid scintillation counting. Non-specific, background level of \textsuperscript{14}CO\textsubscript{2} incorporation was determined by carrying out the above reaction either in the absence of exogenous vitamin K or in the presence of three to four-fold excess of the vitamin K antagonist, warfarin (Endo Laboratories), which inhibits the CAM \gamma-glutamyl carboxylase activity (Tuan 1979).

Protein concentrations were determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Embryonic development and skeletal formation

In a previous investigation (Tuan 1980a), it was observed that embryos
maintained in shell-less culture are substantially retarded in their gross development as compared to normal embryos developing in ovo. In the present study, to assess the effect of shell supplementation in vitro, the Hamburger-Hamilton developmental stages, (Hamilton, 1952) of N, SL, and SS embryos were determined using beak and third toe lengths as the primary parameters. The data for 18-day embryos (Fig. 1) reveal a significant difference in embryonic development as a result of shell supplementation to the cultures. Both parameters indicate that the SS embryos are intermediate in development between the N and SL embryos: N embryos, stage 42; SS, stage 41; and SL, stage 40. It should be pointed out that this observed shell-mediated improvement in development was based on gross growth parameters, specifically the length of appendages. In fact, examination of internal organs showed that maturation of various organs appeared to be comparable in all embryos. Thus, it appears that the primary value of shell supplementation in vitro might be to increase skeletal growth. This supposition was tested by directly comparing the size, weight, and degree of calcification of individual bones isolated from 18-day N, SS, and SL embryos. The results for four sets of bones, calvaria and mandible (both membrane bones), and tibia and femur (both endochondral bones) are presented in Tables 1 (A & B). Table 1A shows that, in all cases, the bones of cultured embryos (both SL and SS) were diminished in weight and size, consistent with the calcium-deficient states of these embryos.

Fig. 1. Gross development of 18-day N, SS and SL embryos as determined by Hamburger-Hamilton staging. The staging parameters used were A) length of third toe and B) beak length. The standard curves are generated from data presented by Hamburger & Hamilton (1951). Each of the values shown represents mean±S.E.M. from measurements on 10–15 embryos.
Table 1. Effect of shell supplementation on embryonic skeletal formation*

<table>
<thead>
<tr>
<th>Embryo</th>
<th>N</th>
<th>SL</th>
<th>SS</th>
<th>SS:SL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Wet weight of bones (mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calvaria</td>
<td>27-3 ± 1-4</td>
<td>17-6 ± 2-3</td>
<td>16-2 ± 2-7</td>
<td>0-92</td>
</tr>
<tr>
<td>Mandible</td>
<td>37-9 ± 3-6</td>
<td>26-1 ± 3-9</td>
<td>29-4 ± 1-8</td>
<td>1-12</td>
</tr>
<tr>
<td>Femur</td>
<td>74-3 ± 7-9</td>
<td>34-6 ± 3-2</td>
<td>43-0 ± 4-5</td>
<td>1-24**</td>
</tr>
<tr>
<td>Tibia</td>
<td>136-5 ± 6-7</td>
<td>46-6 ± 6-2</td>
<td>64-3 ± 8-5</td>
<td>1-38**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Length of bones (mm)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo</td>
<td>N</td>
<td>SL</td>
<td>SS</td>
<td>SS:SL</td>
</tr>
<tr>
<td>Total</td>
<td>15-0 ± 0-7</td>
<td>11-2 ± 0-1</td>
<td>11-6 ± 0-3</td>
<td>1-04</td>
</tr>
<tr>
<td>Femur</td>
<td>8-3 ± 0-4</td>
<td>5-5 ± 0-2</td>
<td>6-1 ± 0-2</td>
<td>1-11**</td>
</tr>
<tr>
<td>Mineralized midshaft†</td>
<td>20-9 ± 1-2</td>
<td>14-2 ± 1-7</td>
<td>15-2 ± 0-8</td>
<td>1-07</td>
</tr>
<tr>
<td>Total</td>
<td>11-2 ± 0-3</td>
<td>6-5 ± 0-6</td>
<td>7-8 ± 0-5</td>
<td>1-20**</td>
</tr>
<tr>
<td>Tibia</td>
<td>20-9 ± 1-2</td>
<td>14-2 ± 1-7</td>
<td>15-2 ± 0-8</td>
<td>1-07</td>
</tr>
<tr>
<td>Mineralized midshaft†</td>
<td>11-2 ± 0-3</td>
<td>6-5 ± 0-6</td>
<td>7-8 ± 0-5</td>
<td>1-20**</td>
</tr>
</tbody>
</table>

* The bones were dissected out from 18-day embryos (N, normal in ovo development; SL, shell-less culture; and SS, shell-supplemented culture). Values represent mean ± S.E.M. from over 10 embryos in each set. Data for SS:SL were compared by Student's t test ** P values <0-05 were considered as significant.
† The region of mineralized midshaft in the long bones was identified either by direct observation of the fresh specimen with transillumination or by histochemical staining of fixed specimen using Alizarin Red.

relative to the normal embryos. It is noteworthy that the endochondral long bones (femur and tibia) were affected relatively more severely than the membrane head bones (calvaria and mandibles). Furthermore, a direct comparison between bones of the SL and SS embryos showed that, upon shell supplementation, the endochondral bones increased significantly in weight, whereas no change was observed in the membrane bones. This observation is indeed consistent with the known temporal sequence of ossification in the developing chick embryo. Although initiation of ossification in the membrane and endochondral bones both begin at approximately the same time (incubation day 7–8), calcification of the former is more or less complete by incubation day 16 whereas the latter continue to mineralize up to hatching (Romanoff & Romanoff, 1949). The results here would indicate that the exogenous eggshell was only partially able to meet the calcium needs of embryonic skeletal mineralization and preferentially supplements those bones whose mineralization persists throughout late development. That skeletal calcification was specifically affected by eggshell supplementation was shown by comparing the lengths (total and mineralized midshaft region) of the respective long bones of the N, SL, and SS embryos. The data in Table 1B
demonstrate that although the overall lengths remained similar, a significant increase in the size of the calcified midshaft region resulted upon shell supplementation and probably accounted for the corresponding increase in weight of the SS bones.

The mobilization of the supplemented eggshell calcium was studied using $^{45}$Ca-labelled eggshell and tracing the distribution of the radioisotope within the embryo as a function of development. Radiolabelling of eggshell pieces by means of equilibration with $^{45}$CaCl$_2$ resulted in significant incorporation of radioactivity which did not appear to be due only to entrapment since repeated rinsing of the treated shell produced a consistent level of approximately 1000 c.p.m./mg dry weight which probably represented genuine ionic exchange with the shell mineral. The results in Fig. 2A indicate that the cultured embryo was able to continuously derive $^{45}$Ca from the added eggshell throughout development. It is interesting to note that addition of eggshell pieces proved to be a convenient and safe method of calcium repletion to the cultured embryos. Initial experiments using various regimens of introducing ionized calcium solutions (e.g. chloride, citrate, acetate) invariably resulted in severe haematomas and eventual death of the embryo within several hours. Similar toxic effects of calcium salts on chick embryos incubated in ovo were also observed by Grabowski (1966). Using the $^{45}$Ca-labelled eggshell, it was also possible to trace the tissue distribution of the supplemented shell calcium in the embryo during development (Fig. 2B). These data show that: (1) After shell supplementation, the long leg bones of the developing embryo exhibited a progressively higher accumulation of $^{45}$Ca compared to other tissues; and (2) A substantial amount of the accumulated $^{45}$Ca was found in the CAM shortly after shell supplementation and, as a function of development, the CAM $^{45}$Ca content rapidly decreased compared to other embryonic tissues. The latter kinetic profile is consistent with the role of the CAM as a tissue which mobilizes and transports extraembryonic calcium into the embryo.

Taken together, the results of these studies above demonstrate that eggshell addition to the cultured embryos partially supplemented the calcium requirement of embryonic development resulting in improved overall growth and increased calcification of the skeletal components, especially the endochondral long bones.

**CAM functions**

The ability of the cultured embryos to acquire calcium from exogenously supplemented eggshell indicated that their CAM must be functional in calcium uptake and transport. Since it was previously observed that the CAM of totally shell-less cultures were severely deficient in calcium transport activity (Tuan, 1980a), the present finding suggests that the functional state of the CAM of SS embryos might have been altered as a result of the presence of the added eggshell. The nature of the eggshell-mediated influence was investigated by directly comparing the calcium transport and related functions of the CAMS of N, SS, and SL embryos. In the SS embryos, two different regions of the CAM were
Fig. 2. (A) Accumulation of supplemented shell calcium by cultured chick embryos during development. Embryos were obtained at various stages of development from cultures which had been supplemented on incubation day 11 (arrow) with $^{45}$Ca-radiolabelled eggshell, dissected clean from extraembryonic tissues, ashed (650°C, 48 h), and prepared for liquid scintillation counting. Estimation of calcium accumulation from the supplemented shell was based on the specific radioactivity (mean = 1000 c.p.m./mg dry weight) of the eggshell piece labelled with $^{45}$Ca as described in text. The average eggshell piece added to the cultures weighed approximately 0.5 g. (B) Tissue distribution of supplemented shell calcium as a function of chick embryonic development. The cultured embryos were supplemented with $^{45}$Ca-labelled eggshell on incubation day 11 (arrow) as described above. The radioactivity accumulated in various tissue parts (○—○, head; ■—■, legs; ▲—▲, trunk and wings; ○—○, CAM) was determined by liquid scintillation counting and expressed as percentages of the total accumulation by the embryo.
tested: the area directly under and adhering to the added eggshell piece (SS-a) and the surrounding nonadherent area (SS-na). The data presented in Fig. 3 were obtained from 17-day embryos and show that: (1) CAM calcium transport activity was significantly suppressed in the SL embryos compared to N embryos as previously reported (Tuan, 1980a); (2) Upon shell supplementation to the cultured embryos calcium transport activity in the CAM was stimulated over twofold and, interestingly, only in the Ss-a region, whereas SS-na regions remained transport-deficient. Furthermore, this increase in transport activity in the SS-a region of the CAM appeared to be a result of and required the continuous presence of the supplemented shell since eggshell pieces added immediately before the transport measurements had no effect. Finally, it was observed that addition of either the shell membrane alone which is highly loaded with calcium carbonate mineral (Plimmer & Lowndes, 1924; Glaser & Pliehler, 1934) or the shell alone, was sufficient to elicit the stimulation of CAM calcium transport in the cultured embryos.

Further investigation was carried out to analyse the biochemical basis of the response of the CAM to the added shell. The CaBP of the CAM was studied in

![Graph showing the effect of supplemented eggshell on CAM calcium transport activity.](image)

**Fig. 3.** Effect of supplemented eggshell on CAM calcium transport activity. All embryos (N, SL and SS) used for comparison were 17 days old. Two regions of the CAM in SS embryos were studied: SS-a, the region directly adhering to the added eggshell; and SS-na, the non-adherent region. Calcium transport activities were based on CAM calcium uptake (nmole/min/cm²) measured as described in Materials and Methods and expressed as percentage values of that in N embryos. The data presented are the mean±s.e.m. of three separate experiments using eight embryos for each determination.
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view of its previously established functional association with the CAM transport function. The CAM samples (N, SL, SS-a, and SS-na) of 17-day embryos were first analysed for their concentrations of the CaBP based on both its calcium-binding activity (Tuan & Scott, 1977) and its immunoreactivity with specific anti-CaBP antibodies (Tuan, 1980a) and the results are presented in Table 2. A comparison between the N and SL embryos reveals that embryonic calcium deficiency due to lack of eggshell resulted in the formation of an inactive form of the CaBP in the CAM, as reported previously (Tuan 1980a). Upon shell supplementation, calcium-binding activity was significantly enhanced in the SS-a, but not in the SS-na, regions of the CAM. On the other hand, compared to SL embryos, the level of immunoreactive CaBP in the CAM appeared to decrease slightly in the SS-a (not in the SS-na) regions, resulting therefore in a relative twofold elevation in CaBP specific activity. These findings therefore suggest that the presence of the eggshell in the cultured embryo resulted in an increase of the relative abundance of the active form of the CaBP in SS CAM, but only in the area lying directly adjacent to the added shell.

It was therefore of interest to investigate the mechanism underlying the apparent activation (or decreased inactivation) of the CaBP in the SS-a CAM. For this purpose, the enzyme activity of vitamin K-dependent \(\gamma\)-glutamyl carboxylase was assayed in microsomes isolated from N, SL, SS-a and SS-na CAM samples. The rationale for assaying this enzyme activity is that in previous investigations it has been shown that the CaBP of the CAM is a vitamin K-dependent protein (Tuan, et al., 1978a, 1978c; Tuan, 1979; Tuan, 1980a) and that expression of the CaBP during normal embryonic development is accompanied by and requires a concomitant vitamin K-dependent \(\gamma\)-glutamyl carboxylase activity (Tuan, 1979). The results in Table 3 show that, in 17-day embryos, the level of

<table>
<thead>
<tr>
<th>Source of CAM</th>
<th>Calcium-binding activity %</th>
<th>Immunoreactive CaBP %</th>
<th>Specific activity (Activity/Immunoreactive CaBP) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>100 ± 12.1</td>
<td>100 ± 10.0</td>
<td>100</td>
</tr>
<tr>
<td>SL</td>
<td>40.8 ± 3.2</td>
<td>207 ± 70.0</td>
<td>19.7</td>
</tr>
<tr>
<td>SS-a</td>
<td>63.2 ± 4.6</td>
<td>158 ± 42.2</td>
<td>40.0</td>
</tr>
<tr>
<td>SS-na</td>
<td>34.4 ± 5.4</td>
<td>260 ± 10.0</td>
<td>13.2</td>
</tr>
</tbody>
</table>

*The level of CaBP in the extracts of CAM prepared from 17-day-old N normal, SL (shellless), and SS (shell-supplemented; including SS-a, shell-adhering, and SS-na, non-adherent CAM) embryos was assayed by: 1) calcium-binding activity (units/mg protein) using the Chelex 100 method (Tuan & Scott, 1977); and 2) radial immunodiffusion (\(\mu\)g CaBP/mg protein) using specific anti-CaBP antibodies (Tuan, 1980a). The specific activity of CaBP is expressed as calcium-binding activity/immunoreactive CaBP. All data represent the mean ± s.e.m. of four separate experiments using five embryos in each set and are expressed as percentage values of those of N embryos.
Table 3. Effect of eggshell supplementation on CAM vitamin K-dependent \( \gamma \)-glutamyl carboxylase activity*

<table>
<thead>
<tr>
<th>Source of CAM</th>
<th>Activity (c.p.m./mg)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>401</td>
<td>100</td>
</tr>
<tr>
<td>SL</td>
<td>256</td>
<td>63.8</td>
</tr>
<tr>
<td>SS-a</td>
<td>366</td>
<td>91.3</td>
</tr>
<tr>
<td>SS-na</td>
<td>168</td>
<td>41.9</td>
</tr>
</tbody>
</table>

*Vitamin K-dependent \( \gamma \)-glutamyl carboxylase activity was assayed as described in Materials and Methods. The activity levels represent the mean of two separate experiments using four to six of each set of embryos (N normal; SL, shell-less, and SS, shell-supplemented). SS-a and SS-na denote CAM of SS embryos adherent or non-adherent to the supplemented shell, respectively. All activities are expressed as percentage values of that in N embryos.

the vitamin K-dependent carboxylase activity, which catalyses the post-translational formation of \( \gamma \)-carboxyglutamic acid residues in the CaBP (Tuan, et al., 1978a), decreased in SL CAM and increased upon shell supplementation in the SS-a, but not in SS-na, region of the SS CAM. Furthermore, it was found that the activity of the microsomally isolated enzyme itself was not affected by the addition or deletion of calcium in the cell-free assay mixture (data not shown). These findings therefore strongly suggest that expression of the vitamin K-dependent enzymatic function in the CAM cells may be directly influenced by the proximity of the CAM to the added shell mineral.

In addition to directly confirming the function of the eggshell as the principal calcium source for the developing chick embryo, the experiments described here have shown that the eggshell plays a stringent regulatory role in the expression of the calcium transport and related activities in the CAM of the chick embryo. The attachment of the CAM to the shell/shell membrane during normal development in ovo (around incubation day 10) is likely to be a signal for the onset of calcium transport and related functions in the CAM ectoderm, possibly via the activation of the vitamin K-dependent enzyme(s) and other yet undetermined mechanism(s). The natural overlay of the eggshell (shell membrane only at the air-space) on top of the entire CAM in ovo should therefore guarantee a development-specific, total activation of the CAM calcium transport function. Since the shell-induced response is localized solely to the region of CAM directly adjacent to the shell/shell membrane, the primary mechanism of action which remains to be elucidated is unlikely to be an entirely humoral one, although the required vitamin K is presumably supplied from the yolk (Bolton, 1961) via the circulatory system. In particular, it is tempting to speculate that the extracellular eggshell/shell membrane mineral matrix is capable of inducing cytodifferentiation of CAM ectodermal cells into a transport-competent cell type and that this matrix–cell interaction operates by means of a short-range (molecular) signal. It
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is noteworthy that among the vast number of studies utilizing the popular technique of tissue grafting on the CAM are at least several (Eisenstein, Sorgente, Soble, Miller & Kuettner, 1973; Jakob, Jentzsch, Mauersberger, & Heder, 1978; Krukowski & Kahn, 1980) that report the CAM as being highly reactive to exogenously added eggshell/shell membrane material. These workers have observed local, inflammation-like responses in the CAM ectoderm (and underlying mesoderm) in contact with the eggshell, followed by formation of giant cells, proliferation of blood vessels and fibroblasts, and invasion of cellular processes into the shell matrix. Whether these cellular events are involved in the eggshell-mediated restoration of calcium transport in the CAM remains to be investigated. Finally, since our findings show that eggshell pieces alone are also effective in restoring calcium transport and related functions to the CAM, the nature of the mineral matrix required is a question worthy of consideration. It should be of interest to assess the effectiveness of different forms of calcite derived from calcified eggshells or tissues of other species or from inorganic ores.

The studies presented here illustrate the uniqueness of the shell-less chick embryo culture as it offers a means of experimentally modulating the metabolic calcium supply to the growing embryo and as such allows one to manipulate expression of a developmental function. It is interesting to point out that although several morphological studies (Narbaitz & Jande, 1978; Dunn & Fitzharris, 1979) have found no apparent difference in the gross cytohistology of the CAM between N and SL embryos, they are functionally distinct in that SL CAM was found to be deficient in calcium transport (Tuan, 1980a). In fact, in a recent study, Dunn, Graves & Fitzharris (1981) confirmed this observation and furthermore reported that addition of eggshell pieces to the CAM during culture also partially restores the calcium transport capacity as measured in vitro by the Ussing chamber technique. These corroborative findings, taken together, strengthen the hypothesis that expression of the calcium transport function in the CAM is under stringent regulation by the transport substrate, the eggshell, itself. The biochemical data presented here show that one of the key components in the CAM transport pathway under eggshell-mediated regulation is the CaBP whose activation appears to be intimately linked to the proximity of the eggshell to the CAM. Although the precise mechanism controlling CaBP activation is not known, these data suggest that it may depend on the functional state of the vitamin K-dependent carboxylation system in the CAM. It needs to be pointed out that in the microsomal carboxylation assay used here total endogenous carboxylation was measured. Hence a change (increase or decrease) in measured carboxylation activity may reflect changes in availability of endogenous substrate(s) and/or changes in the intrinsic activity level of the carboxylase enzyme. The exact involvement of these parameters as well as the identity of the carboxylated protein(s) in the CAM microsomes are currently being investigated.
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