Transepithelial calcium transport in the chick chorioallantoic membrane

I. Isolation and characterization of chorionic ectoderm cells

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SUMMARY

The chicken eggshell supplies approximately 80% of the calcium found in the hatchling chick. The mobilization of eggshell calcium into the developing embryo involves the transepithelial transport of large amounts of calcium in a development-specific manner. The cells responsible for the transport of eggshell calcium into the embryonic circulation are the ectodermal cells of the chorioallantoic membrane. In this report, we present a method for the isolation and culture of chorioallantoic membrane ectodermal cells, which are amenable to direct experimental manipulation. Cell preparations are characterized with respect to the expression of an ectoderm-specific cell surface marker (transcalcin, a calcium-binding protein), and a specific enzymatic activity (elevated Ca²⁺-activated ATPase). Functional assessment of in vitro cellular calcium uptake by ⁴⁵Ca²⁺ tracer kinetics indicates the persistence of a temperature-sensitive, rapid-influx pathway similar to that observed in vivo. The preparations of primary ectodermal cells present an in vitro system applicable to the experimental analysis of calcium metabolism and transport by the chick chorioallantoic membrane.

Key words: chick embryo, epithelium, active transport, Ca-ATPase, calcium binding protein, egg shell membrane

INTRODUCTION

Calcium is a major nutritional requirement for the developing chicken embryo. As extensive mineralization of the embryonic skeleton begins, after day 10 of incubation, large amounts of calcium are mobilized from the calcite stored in the eggshell. Nearly 80% of the approximately 140-180 mg of calcium in the day 20-21 hatching chicken is derived from the eggshell (Johnston and Comar, 1955; Romanoff, 1961) and is transported into the embryonic bloodstream by the chorioallantoic membrane (CAM). In this report, we present a method for the isolation and culture of chorioallantoic membrane ectodermal cells, which are amenable to direct experimental manipulation. Cell preparations are characterized with respect to the expression of an ectoderm-specific cell surface marker (transcalcin, a calcium-binding protein), and a specific enzymatic activity (elevated Ca²⁺-activated ATPase). Functional assessment of in vitro cellular calcium uptake by ⁴⁵Ca²⁺ tracer kinetics indicates the persistence of a temperature-sensitive, rapid-influx pathway similar to that observed in vivo. The preparations of primary ectodermal cells present an in vitro system applicable to the experimental analysis of calcium metabolism and transport by the chick chorioallantoic membrane.

Key words: chick embryo, epithelium, active transport, Ca-ATPase, calcium binding protein, egg shell membrane
cium transport, the CC cells develop long, thin cytoplasmic extensions that form a barrier between the eggshell calcium reserve and the embryonic circulation (Coleman and Terepka, 1972a-c). As calcium transport increases, the cytoplasmic extensions become extensive and narrow so that the capillaries approach the eggshell calcium reserve more closely (Coleman and Terepka, 1972c). By day 15, prior to the peak of transport activity (Crooks and Simkiss, 1975; Terepka et al., 1969; Tuan, 1980), the CC cells contain few membranous organelles, and lack endoplasmic reticulum and Golgi apparatus in particular (Coleman and Terepka, 1972c). In conjunction with these distinct morphological changes, the CAM ectoderm expresses biochemical activities associated with calcium transport, including a vitamin K-dependent Ca\(^{2+}\)-binding protein (transcalcin) (Tuan, 1987; Tuan et al., 1978a-c; Tuan and Scott, 1977), and an enhanced Ca\(^{2+}\)-activated, Mg\(^{2+}\)-dependent ATPase activity (CaATPase) (Tuan and Knowles, 1984). Each of these biochemical activities has been functionally linked to CAM Ca\(^{2+}\) transport in vitro and in vivo (Tuan et al., 1986b).

The relatively simple cytoarchitecture of the CAM, and the availability of specific markers, make CC cells ideal candidates for the in vitro assessment of transepithelial calcium transport; however, as pointed out by previous researchers, there are several difficulties related to tissue inaccessibility and the presence of the adherent ISM (Gar- rison and Terepka, 1972a; Terepka et al., 1969). In order to delineate the cellular pathway involved in the transcellular mobilization of calcium across the CAM ectoderm, we sought to obtain cell preparations suitable for in vitro analyses and manipulations. We present here a straightforward method for the preparation of the CAM ectoderm cells implicated in calcium transport. These cells are isolated specifically from the CAM ectoderm and retain surface transcalcin expression, elevated CaATPase activity, and the rapid single-step calcium influx pathway characteristic of CAM ectoderm in vivo. Our results indicate that isolated primary ectoderm cells may be useful for the in vitro characterization of the calcium transport function of the CAM.

**MATERIALS AND METHODS**

**Chick embryo and shell-less embryo culture**

Fertilized chicken embryos (Truslow Farms, Chesterfield, MD) were kept at 37.5°C in a humidified egg incubator for the number of days desired. Shell-less embryos were prepared on incubation days 3 and cultured as described by Tuan (1980) after Dunn and Boone (1976).

**Preparation of tissue discs**

The CAMs of normal embryos incubated in ovo were prepared for cell isolation by first preparing tissue discs. The egg surface was disinfected with 70% ethanol, and the eggshell was broken above the air-space to expose the outer surface of the ISM. A section of the exposed ISM and underlying CAM were cut and removed as were the embryo, yolk, albumen and allantoic fluid. The bulk of the CAM remained attached to the eggshell. After being cut into halves, the eggshell was carefully separated from the shell membranes and CAM. The tissue was placed, ISM side up, on Parafilm (4 layers thick) and cut into 2 cm\(^2\) circles, ‘cookie cutter style’, with a sharp cork-borer. The resulting tissue discs were placed in ice-cold Hanks’ Balanced Salt Solution with 10 mM HEPES (pH 7.2) (HBSS) until the shell membranes were thoroughly wetted. The CAM discs were carefully separated from the ISM using fine forceps and transferred to a fresh container of cold HBSSH.

**Sample preparation for transmission electron microscopy (TEM)**

CAM tissue samples were processed for electron microscopy as described by Tuan et al. (1986a). Briefly, tissue was removed from embryos, rinsed in ice-cold HBSSH and then iced-cold 0.12 M sodium cacodylate (pH 7.4). Samples were fixed overnight in 0.25% glutaraldehyde (EM Science, Cherry Hill, NJ) in cacodylate buffer. Samples were rinsed, post-fixed for 1 hour and embedded in Epon. Sections were stained with lead and uranyl acetate and viewed using a JEOL 100B transmission electron microscope.

**Sample preparation for scanning electron microscopy (SEM)**

Tissue specimens were mounted into O-rings with the ectodermal surface up and rinsed in HBSSH followed by ice-cold 0.12 M sodium cacodylate buffer (pH 7.4). Tissue was then fixed overnight with 0.25% glutaraldehyde (EM Science) in the same buffer, rinsed, post-fixed for 1 h with 1% OsO\(_4\), rinsed, dehydrated through ethanol, and critical point dried into 100% CO\(_2\). The samples were mounted onto aluminum stubs, sputter-coated with gold, and observed using a JEOL 35-C scanning electron microscope.

**Isolation and culture of CAM ectoderm cells**

Embryos were dissected as described above for tissue disc preparation. After removal of the eggshell, the CAM tissue, with shell membranes attached, was rinsed in room temperature HBSSH until the ISM began to separate from the CAM. Small pieces of ISM were left in place to mark the chorionic side, and the tissue was drained and placed onto a 0.45 μm pore size, UV-sterilized, nitrocellulose (NC) sheet (Millipore, Bedford, MA) with the wetted shell membranes-facing upward. After the allantoic cells had thoroughly adhered to the NC, the CAM was peeled away leaving the allantoic cells behind. The tissue was returned to HBSSH and the remaining pieces of ISM removed. The CAM fragment was then minced with scissors and digested with 0.1% trypsin (Sigma, St. Louis, MO), 10 mM EDTA (Sigma) in HBSSH at 37°C in a stirring flask. After 30 min, aliquots of digested material were assayed every 10 min using a hemocytometer. When numerous small clumps of cells (5-10 cells per clump) were evident, the digestion was stopped by the addition of soybean trypsin inhibitor (Sigma) and 0.05% deoxyribonuclease (Sigma) for 15 min at 37°C. Tissue remnants were removed, and the suspended cells were centrifuged at 300 g in an IEC (Boston, MA) centrifuge for 20 min to collect the ectoderm cells. The cell pellet was resuspended in 10% calf serum, 5 μM hemin, Ham’s F-12 medium ( Gibco-BRL, Gaithersburg, MD) to a total cell density of 1x10\(^6\) to 2x10\(^6\)/ml. Fibroblasts were allowed to adhere to tissue culture dishes for 30 min at 37°C, then the non-adherent cells were plated, 1 ml of cells per 35 mm diameter well, on dishes coated with Matrigel (1/10 diluted; Collaborative Research, Bedford, MA) and cultured at 37°C with 5% CO\(_2\).

**Sample preparation for light microscopy**

**Tissue fixation and sectioning**

Tissue sections for immunofluorescence were prepared from day 14 normal CAM. The tissue was prepared as described previously (Ono and Tuan, 1991). Briefly, pieces of CAM were fixed in a
modified Carnoy’s fixative, embedded in paraffin, sectioned at 8 μm thickness, cleared and rehydrated. Tissue sections for hematoxylin and eosin staining were similarly prepared except that fixation was carried out in Bouin’s fixative (Humason, 1967).

**Cryosectioning and ATPase histochemistry**

Cryosectioning of CAM from day 14 normal embryos was removed and frozen-embedded at –70°C in Tissue-Tek O.C.T. Compound (Miles Inc., Diagnostics Div.; Elhart, IN). Sections were cut at 12 μm thickness and –20°C using a Tissue-Tek microtome (Miles Inc.; Naperville, IL), then fixed for 30 s in –20°C acetone, and air-dried on glass microscope slides. CaATPase histochemistry was carried out as previously described (Tuan and Knowles, 1984) after the method of Ando et al. (1981).

**Immunofluorescence and immunohistochemistry**

Antiserum to the CAM-specific calcium-binding protein, transcaldesmin (Tuan et al., 1978a; Tuan, 1987), was thawed from –20°C using a Zymed HISTOSTAIN-SP kit (with TBSTSB substituted for PBS in antibody and rinse solutions). After PBS in antibody and rinse solutions, sections were incubated for 30 min at room temperature. Samples were rinsed extensively with TBSTSB and then twice with TBS. Sections were mounted in glycerol-vinyl-ethanol mounting medium (Olympus BH-2 microscope with epi-illumination fluorescence).

**Calcium transport. I. Isolation of cells**

Calcium uptake assays

**Calcium and inulin uptake by isolated tissue discs**

Calcium and inulin uptake by isolated CAM ectoderm cells were carried out using a modification of a procedure described previously for CAM microsomal calcium uptake (Tuan et al., 1986a). Briefly, cells were isolated and cultured as described above. Cells were released from tissue culture dishes into suspension using a non-enzymatic cell dissociation solution (Sigma), and collected by centrifugation at 300 g in an IEC centrifuge for 20 min. Cell pellets were resuspended into Hanks’ Balanced Salt Solution with HBSS at the temperature indicated in the text, and the cells were dispersed. Cell densities were determined using a hemocytometer. At time zero, calcium was added to the cell suspension (final concentration of 0.1 or 1.0 mM as indicated) with 45Ca++ as a tracer; at various time points, aliquots of the cell suspension were centrifuged at 1.04 g/cm^3 sp. gr. mineral oil (Dow Corning) to pellet the cells. 45Ca++ accumulation was determined by liquid scintillation counting. In some experiments, the harvested cells were exposed to either 1 mM EGTA or 2 mM CaCl2 to remove or compete external 45Ca++ label prior to collection. Calcium flux was determined either by direct calculation of the difference between calcium uptake at 30 s and 2 min or by indirect calculation from coefficients determined by mathematical curve fitting of the kinetic profile.

**Data analysis**

Curve fitting was carried out using Enzfitter software (Leatherbarrow, 1987) and matched to the general equation: uptake = (A/λ1)1−(e−λ1t)+B(λ23)1−(e−λ2t)+... , where A, B, λ1, λ2,... are constants and each additional term represents the mathematical values of various cellular compartments that may be involved in calcium uptake. Curve fit data were used to calculate the calcium flux rates and compartment sizes of the system after the method of Borle (1990) and using the equations derived by Uchikawa and Borle (1981). Briefly:

For calcium uptake from the extracellular fluid into a single compartment (simple):

\[ \rho_1 = A, \]
\[ S_1 = A/\lambda_1, \]

for calcium uptake into two cellular compartments in series (cannery):

\[ \rho_1 = C_e (A + B) / C_T, \]
\[ S_1 = \rho_0 + [\lambda_1 + \lambda_2 - (A + B)/C_T] - (\lambda_1 \lambda_2)/(A + B)], \]
\[ \rho_2 = S_1 [([\lambda_1 \lambda_2 C_e/(A + B)] - C_e \lambda_1 \lambda_2 / C_T \lambda_1 + \lambda_2 - (A + B)/C_T) - (\lambda_1 \lambda_2)/(A + B))], \]
\[ S_2 = S_1 [([\lambda_1 C_e \lambda_1 \lambda_2]/(\lambda_1 + \lambda_2 - (A + B)/C_T)] - (\lambda_1 \lambda_2)/(A + B))]; \]

for calcium uptake into two cellular compartments in parallel (mammillary):

\[ X = \lambda_1 + \lambda_2 - (A + B)/C_T + [\lambda_1 + \lambda_2 - (A + B)/C_T]^2 - (4 \ C_e \lambda_1 \lambda_2 / C_T]^3]/2, \]
\[ \rho_1 = C_e \ [X(\lambda_1 + \lambda_2) - X + (C_e \lambda_1 \lambda_2 / C_T)X] - \lambda_1 \lambda_2 + (C_e \lambda_1 \lambda_2 / C_T) / [X - (C_e \lambda_1 \lambda_2 / C_T)X], \]
\[ \rho_2 = (C_e \lambda_1 \lambda_2 / C_T)X, \]
\[ S_1 = \rho_1 X, \]
\[ S_2 = \rho_2 C_T X/(C_e \lambda_1 \lambda_2), \]

where \( \rho \) represents steady-state calcium flux rate in nmol/h for either 10^6 cells or 1 cm^2 of ectodermal surface area; \( S \) represents compartment size in nmoles of calcium in either 10^6 cells or 1 cm^2 of ectodermal surface area; \( C_e \) is the external medium calcium; \( C_i \) is the calcium in cellular compartment(s); and \( C_T \) is the total calcium at the end of the assay, in nmoles of calcium per
10^6 cells or 1 cm² of ectodermal surface area. In the case of two compartment mammillary uptake, the intermediate value, \( \lambda \) (equivalent to the per h rate constant of calcium flux from compartment one into the medium), was calculated prior to determining the other values.

**RESULTS**

To begin our study, we first confirmed the sparse distribution of cytosolic structures in CAM CC cells by TEM. Fig. 1A shows the ultrastructure of a section of day 14 CAM tissue including the apical region of a CC cell; consistent with previous descriptions (see Introduction), the cytosol of the CC cell appeared sparse with few membranous organelles. The general structure of the CAM/ISM inter-

![Fig. 1](image_url)

face, as seen by TEM, is shown diagrammatically in Fig. 1B.

To isolate calcium-transporting ectodermal cells from the chick CAM, it was necessary: first, to separate the cell layer from two adherent, acellular structures (i.e. the ISM and the basement membrane), and next to purify the CC cells from several contaminating cell types (i.e. allantoic cells, fibroblasts, blood cells, endothelial cells, etc.). We initially sought to release the CAM from the ISM by enzymatic digestion, but found that it was equally effective to simply soak the ISM with buffered saline until the two layers separated, and the coloration of the ISM turned from white to gray. The result of wetting the ISM prior to removal from the CAM is summarized in the SEM micrographs of Fig. 2. Fig. 2A represents the ectodermal CAM surface of a

![Fig. 2](image_url)
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The CAM of the shell-less embryo was never attached to the ISM and, therefore, served as a reference for the general morphology of the intact ectodermal layer. The shell-less CAM preparation showed a distinctive, cobblestone-like pattern of ectoderm cells. Fig. 2B shows a normal CAM removed from the ISM without wetting. The delicate CC cells were clearly destroyed and the extensive CAM capillary network exposed, a result consistent with the reported loss of calcium transport function upon mechanical removal of the ISM (see Introduction). Fig. 2C shows a CAM prepared as described above with the ISM thoroughly wetted before removal. The CC cells appeared intact, compared to the cells seen in Fig. 2B, and appeared similar to those of the SL embryo shown in Fig. 2A. Large CC cells were seen surrounding VC cells in a cobblestone-like arrangement with approximately 10^6 CC cells per cm^2 of ectoderm surface.

We also assessed functional differences in calcium uptake between normal CAM tissue discs, prepared with or without wetting of the ISM prior to its removal. As shown in Fig. 3, a fluid-phase marker, [3H]inulin, was used in combination with ^45Ca^{2+} to examine the extent to which tissue disc preparations accumulated calcium in a specific manner. The data showed that the rate of inulin accumulation by CAM tissue discs was the same with or without intact ectoderm. Calcium uptake by CAM tissue discs with disrupted ectoderm followed kinetics similar to inulin uptake, indicating that, in this case, calcium uptake was largely accounted for by a fluid-phase component alone. On the other hand, tissue discs with intact CAM ectoderm accumulated calcium in a manner that was consistent with a specific calcium-uptake mechanism like that seen in ovo (Crooks and Simkiss, 1975), where sustained and elevated levels of uptake above fluid-phase levels were evident. The morphological and physiological properties of these CAM tissue discs thus demonstrated the usefulness of such preparations for the further isolation of calcium transport cells or for direct, in vitro experimentation.

To prepare CAM ectoderm cells for experimental analysis, CAM tissue discs were further processed and digested. After careful removal of the hydrated ISM, the tissue was drained, and allantoic derivative endoderm cells were removed by placing the tissue, allantoic side down, onto NC. The endoderm adhered to the NC and the ectoderm could be peeled away. This peeling procedure cleaved the tissue down the mesodermal layer and left the ectodermal

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**Fig. 2.** Scanning electron micrographs of the eggshell-facing portion of the chick CAM after different treatments. CC, capillary covering cells; VC, villus cavity cells; EN, endothelial cells; D, cell debris. (A) CAM ectoderm cells of a day 14 shell-less embryo. These cells were not allowed to attach to the ISM during development and, consequently, exhibit the general morphology of undisturbed CAM ectoderm cells. (B) CAM ectoderm cells of a day 14, normal chick embryo with the ISM removed without prior hydration. The destructive effects of directly separating the CAM and ISM are evident; the endothelial cells of the extensive CAM capillary network are clearly visible along with VC cells and the debris of the destroyed CC cells. (C) The eggshell-facing surface of CAM ectoderm cells of a day 14 normal embryo with the ISM removed after rehydration as described in Materials and Methods. Several large CC cells were seen surrounding VC cells, such that 1 cm^2 contained approximately 10^6 cells. Although the cytoplasmic extensions of these CC cells appeared more extensive, the cobblestone morphology of this surface was essentially similar to the shell-less ectoderm (see Fig. 1A). Bar, 10 µm.
cell layer intact. Fig. 4A shows a histological section of a CAM sample immediately after dissection from the embryo with the attached ISM. Fig. 4B shows a similar piece of tissue after removal of the ISM and stripping of the allantoic derivative cells. The chorionic ectoderm cells were subsequently prepared for culturing by partial enzymatic digestion of the tissue fragments that remained after ISM separation and endoderm removal. To avoid contamination of the culture by large numbers of capillary endothelial cells or fibroblasts, samples were digested with trypsin/EDTA until small clumps of 5 to 10 cells became evident. The ectoderm cells were separated from the remaining tissue fragments, collected and fibroblasts were removed by pre-plating onto tissue culture plastic.

To characterize the CAM cell preparations, the degree to which isolated cells maintained markers and activities characteristic of the CAM ectoderm was assessed. As shown in Fig. 5, the cell surface calcium-binding protein, transcalcin, localized to the CAM ectodermal region. CaATPase cyto-histochemistry, as shown in Fig. 6, demonstrated that the cells of the CAM ectoderm contain a high level of activity relative to the surrounding tissue. Transcalcin also localized to a subset of cultured CAM cells that had been isolated by extensive digestion of whole CAM (i.e. without prior removal of the allantoic-derivative, endoderm cells). As shown in Fig. 7A, the mixed population of cells released from the intact CAM contained an expectedly small subset of cells that were strongly positive for transcalcin. In Fig. 7B, a similarly stained culture of preferentially isolated CAM ectoderm cells is shown; a high proportion (≥ 80% in each preparation) of cells with surface transcalcin were seen by immunohistochemistry.

We also examined the distribution of CaATPase activity in cultured CAM cells. Fig. 8A shows histochemical staining of a mixed cell population isolated by extensive digestion from intact CAM. Various levels of CaATPase activity were seen; a small subset of the cells were intensely stained. Fig. 8B shows that, in the enriched preparation of CAM ectoderm cells, most of the cells exhibited intense CaATPase activity staining relative to the few remaining fibroblasts. The observation of two distinct CAM ectoderm markers, cell surface transcalcin and elevated CaATPase activity, clearly indicated that cells prepared by the selective isolation procedure described in this report represent the population of CAM ectoderm cells presumed to be responsible for eggshell calcium transport.

To ascertain functional similarities between cells in vitro and the CAM in vivo, we analyzed and compared the kinet-
ics and temperature dependence of calcium uptake activity in circular pieces of CAM tissue and in isolated CAM ectodermal cells. Using our observation that the ectodermal face of the CAM was made up of approximately $10^6$ cells per cm$^2$ of surface area, we compared the results of our in vitro characterizations to CAM calcium uptake seen in ovo. Fig. 9A depicts the kinetic profiles of calcium uptake by embryonic CAM tissue in ovo, CAM tissue discs, and isolated CAM ectodermal cells at 37°C. The uptake for all three preparations followed similar kinetics: rapid accumulation reaching a plateau in about 15 min. Decreasing the assay temperature to 23°C had an inhibitory effect on calcium uptake in each type of preparation, and, as shown in Fig. 9B, the initial rate of $^{45}$Ca$^{2+}$ accumulation was directly dependent on assay temperature.

Since information derived from the kinetic profiles of $^{45}$Ca$^{2+}$ uptake can be analyzed to assess the compartmentalization of Ca$^{2+}$, we used computer curve fitting analysis to look at the routes of calcium uptake in each type of CAM preparation. As summarized in Table 1, a single mathematical term best accounted for each uptake curve, i.e. the shapes of the $^{45}$Ca$^{2+}$ accumulation curves in ovo, in tissue discs and in isolated cells were all consistent with a rapid influx of calcium into a single cellular compartment.

Table 1. Computer curve-fitting analysis of calcium uptake for each of the three CAM preparations (in ovo, tissue discs and isolated cells)

<table>
<thead>
<tr>
<th></th>
<th>in ovo</th>
<th>Tissue discs</th>
<th>Cells</th>
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<tbody>
<tr>
<td><strong>Simple uptake</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$A$</td>
<td>148.2</td>
<td>296.8</td>
<td>123.6</td>
</tr>
<tr>
<td>$B$</td>
<td>426</td>
<td>28.8</td>
<td>39.6</td>
</tr>
<tr>
<td>$\lambda_1$</td>
<td>424</td>
<td>143.4</td>
<td>61.8</td>
</tr>
<tr>
<td>$\lambda_2$</td>
<td>425</td>
<td>29.0</td>
<td>37.2</td>
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<tr>
<td>$p_1$</td>
<td>146.4</td>
<td>286.2</td>
<td>121.7</td>
</tr>
<tr>
<td>$p_2$</td>
<td>0.00</td>
<td>0.01</td>
<td>-0.38</td>
</tr>
<tr>
<td>$S_1$</td>
<td>3.48</td>
<td>9.96</td>
<td>3.12</td>
</tr>
<tr>
<td>$S_2$</td>
<td>0.01</td>
<td>0.00</td>
<td>-1.70</td>
</tr>
</tbody>
</table>

Data were analyzed assuming each of three different uptake schemes: flux into a single exchangeable calcium pool (Simple uptake), flux into two exchangeable calcium pools in series (Catenary uptake), and flux into two independent calcium pools in parallel (Mammillary uptake). Raw data were entered into the "Enzfitter" program (Leatherbarrow, 1987) to fit the equation: $U=A/(\lambda_1(1-e^{-\lambda_1t}))+B/(\lambda_2(1-e^{-\lambda_2t}))$. For uptake into a single cellular compartment, $B$ was considered to be zero, and the equation was solved for $A$ and $\lambda_1$ only; otherwise, the four variables were solved to give the best fit to the raw data. Solutions to the exponential equations were combined with the measured quantities of $^{45}$Ca$^{2+}$ used to determine cellular compartment sizes and the corresponding steady state calcium flux rates into each compartment based on equations derived by Uchikawa and Borle (1981) as described in Materials and Methods. $p$ represents steady state calcium flux rate in nmole/h for either $10^6$ cells or 1 cm$^2$ of ectodermal surface area; $S$ represents compartment size in nmole of calcium in either $10^6$ cells or 1 cm$^2$ of ectodermal surface area. Calcium uptake for each of the preparations was most accurately represented by uptake into a single compartment. When a two-compartment, catenary system was assumed, the second compartment in the series contained virtually no calcium ($S_2=0$), and the corresponding flux rates ($p_2$) were either near zero or negative in value. When a two-compartment, mammillary system was considered, the resulting $S_2$ and $p_2$ values were also anomalously negative. Negative or zero values indicated that the model upon which the calculations were based could not be used to fit the observed uptake kinetics. The results of the curve fitting analyses, therefore, indicate that the rapid calcium uptake seen in ovo, in tissue discs ex vivo, and in cultured cells in vitro each occurred into a single cell or tissue compartment.
Fig. 6. Histochemical localization of Ca\textsuperscript{2+}-activated ATPase activity in intact day 14 CAM. The cryosectioned tissue showed that the dark brown reaction product (arrows) was localized exclusively to the ectodermal layer (EC) but not the mesoderm (M). Bar, 50 \( \mu m \).

Fig. 7. Immunohistochemical detection of transcalcin in isolated CAM ectoderm cells. (A) Cells isolated from intact CAM show a small population of cells positive for transcalcin (arrow). (B) After enrichment (see Materials and Methods), cultures of CAM ectodermal cells show a very high percentage of transcalcin positive cells with reddish staining. Bar, 10 \( \mu m \).

Fig. 8. Histochemical detection of Ca\textsuperscript{2+}-activated ATPase activity in cultures of isolated CAM ectoderm cells. (A) Cells isolated from intact CAM digestions showed a low percentage with elevated levels of CaATPase (arrow) as indicated by a brown reaction product. (B) After enrichment (see Materials and Methods), a high level of ATPase activity was seen in most ectodermal cells. Bar, 20 \( \mu m \).
Calcium uptake by the CAM is an important, developmentally regulated function of the chick embryo. Research on the mechanism of CAM calcium transport has been limited due to the lack of suitable in vitro preparations. Previous studies of the physiological and mechanistic aspects of CAM calcium transport have relied on tissue preparations with the ectodermal cells obscured by the maternally derived ISM (e.g. see Crooks and Simkiss, 1975; Crooks et al., 1976; Garrison and Terepka 1972a,b; Terepka et al., 1969, 1976; Tuan, 1980; Tuan et al., 1986a,b; Tuan and Zrike, 1978), fixed and sectioned material (e.g. see Coleman and Terepka, 1972a-c), subcellular membrane preparations (Tuan et al., 1986a,b), or biochemical activities (e.g. see Tuan, 1984; Tuan et al., 1978b,c, 1986a,b; Tuan and Knowles, 1984; Tuan and Scott, 1977; Tuan and Zrike, 1978). We present here a method to isolate and culture CAM ectoderm cells for the investigation of calcium homeostasis at the cellular level. We also demonstrate the in vitro maintenance of a calcium influx pathway that is characteristic of the rapid accumulation of calcium seen in ovo.

The preparation of CAM tissue discs with intact ectodermal surfaces was possible due to the nature of the interaction between the CAM and the ISM. During the course of development, as embryonic respiration increases, the ISM progressively dehydrates (Seymour and Piiper, 1988; Seymour and Visschedijk, 1988). Dehydration of the ISM roughly parallels the increase in its adhesion to the CAM, and, since simple hydration of the ISM prior to removal from the CAM was sufficient to allow the maintenance of the integrity of the CAM ectodermal surface, dehydration may account for CAM to ISM adhesion. Careful removal of the wetted ISM allowed direct access to the CAM ectoderm and made the tissue suitable for further in vitro manipulations.

As shown in Fig. 2, SEM analysis of the ectodermal surface of CAM preparations showed a cobblestone-like pattern of cells in the shell-less chick embryos, similar to that previously reported by Dunn and Fitzharris (1979). When the ISM was hydrated prior to its removal from the CAMs of embryos developing in ovo, a cobblestone-like arrangement of cells was also observed. Although the cells of the shell-less CAM appeared smaller than those of the normal CAM, a difference that may reflect changes in morphology modulated by the presence of the ISM and eggshell (see Coleman and Terepka, 1972c; Tuan, 1983), the preparations were remarkably similar. Tissue discs may prove useful in the characterization of calcium uptake and transport by the CAM in a multicellular, in vitro system that should closely simulate in vivo conditions. It must be emphasized, however, that the underlying endothelial cells also transport calcium, and care must be taken to account for uptake into each cell layer.

Tissue discs of CAM have been shown to exhibit rapid, active and unidirectional calcium transport. The first component of this transport is uptake of calcium across the apical membrane of the CAM ectoderm. Crooks and Simkiss (1975) demonstrated that tracer $^{45}\text{Ca}^{2+}$ accumulation from the eggshell facing surface of the ISM was approximately equal to the accumulation of calcium in the embryo (i.e. net flux into the CAM from the eggshell equals flux out of the CAM into the embryonic compartments). In addition, Terepka and co-workers showed in vitro that the reverse flux of $\text{Ca}^{2+}$ from the CAM back toward the eggshell was nearly negligible and that the CAM continued to accumulate calcium up substantial concentration gradients (see Terepka et al., 1976). Therefore, $\text{Ca}^{2+}$ taken up by the rapid apical pathway of the CAM ectoderm seems destined for transport. Since isolated CAM ectoderm cells...
maintain a rapid Ca\(^{2+}\) uptake pathway similar to that seen in vivo, these cells may represent an exciting in vitro system for analysis of CAM calcium homeostasis.

It should be pointed out that we have attempted to analyze calcium transit through monolayer CAM ectoderm cells cultured on permeable Transwell membranes (Costar; Cambridge, MA), to simulate an epithelial cell layer (see Breitfield et al., 1989). Unfortunately, isolated CAM cells did not proliferate to form confluent monolayers in vitro, and we were unable to establish a suitable transport system. We were also unable to obtain viable, CAM ectoderm cells from embryos older than incubation day 15, although, during development in ovo, transport activity does not peak until around day 17. Both these observations are consistent with the morphological descriptions of ectoderm cells reported by Coleman and Terepka (1972c): the CC cells become terminally differentiated during development as calcium transport activity begins to increase in the CAM. Since mature CC cells lack membranous biosynthetic organelles (Coleman and Terepka, 1972c), the cells may simply be non-proliferative and recover poorly from enzymatic dissociation. Thus, it was not unexpected that the most successful cell isolations were those using embryos between days 12 and 15 of incubation, a period corresponding to the early phase of increasing calcium transport activity (Tuan et al., 1986a), but prior to the complete loss of cellular biosynthetic machinery (Coleman and Terepka, 1972c). In fact, in terms of the developmental profile of calcium uptake activity in isolated cells, we have observed the highest level of activity in day 13 to 15 CAM (data not shown). In this study, CAM cells were not harvested before day 10 of incubation, since CAM calcium transport does not normally start until later (Tuan, 1987). It should be noted that we have recently analyzed the calcium-uptake characteristics of the human choriocarcinoma cell line, JEG-3 (Tuan et al., 1991). The JEG-3 cells are derived from an epithelial choriocarcinoma and appear to exhibit calcium-transport-related activities similar to those of isolated CAM cells. JEG-3 cells may be useful in the direct analysis of transepithelial calcium transport by cells of extra-embryonic membranes.

Taken together, the presence of two known functional biochemical components of calcium transport in the isolated CAM cells, along with their ability to accumulate calcium rapidly in a manner similar to that seen in vivo and in tissue discs in vitro, strongly suggest that the isolated primary CAM ectoderm cells represent the calcium transport component of the CAM choricionic epithelium. The CAM ectoderm cells should, therefore, constitute a valid primary culture system for characterizing the cellular mechanism of CAM calcium transport and investigating calcium homeostasis in this transport epithelium. In the accompanying paper, we have characterized the calcium influx phase of the CAM transepithelial calcium transport pathway by analyzing the cellular distribution of calcium in isolated CAM ectoderm cells during the initial stages of calcium accumulation.

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