Role of Calcium and Calmodulin in Hemidesmosome Formation In Vitro

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ABSTRACT Intact epithelial sheets were removed from rabbit corneas using Dispase II, a bacterial neutral protease. The freed sheets were placed on denuded corneal basal laminae and incubated at 35°C for 3, 6, 18, or 24 h. Epithelial-basal lamina preparations were incubated in culture medium that either contained (*a*) varying concentrations of Ca^{2+} ions, (*b*) calmodulin antagonists, (*c*) exogenous calmodulin following an initial 6-h incubation in the presence of antagonists, or that lacked (*d*) Mg^{2+} ions. Tissues were processed for electron microscopy, and micrographs were taken of basal cell membranes. At least four experiments were conducted for each treatment, and for each experiment the total number of hemidesmosomes were counted along the basal membrane-basal lamina surface of eight cells. The number of hemidesmosomes formed was directly proportional to the increasing concentration of Ca^{2+}. The presence or absence of Mg^{2+} ions did not change the numbers of hemidesmosomes formed. Calmodulin antagonists inhibited hemidesmosome formation, and this inhibition was reversed by the addition of calmodulin. Thus, hemidesmosome formation is Ca^{2+} dependent and appears to be mediated by a calmodulin-regulated mechanism.

Adhesion of one cell to another or to a substrate is a fundamental property of cells in higher organisms. Divalent cations such as Ca^{2+} play a major role in many diverse cell-cell adhesion systems. Grunwald et al. (1) demonstrated that dual adhesion mechanisms existed in disassociated embryonic neural retina cells, and Brackenbury et al. (2) reported that the phenomenon was also true for both neural and non-neural tissue throughout the chick embryo. A dual adhesion mechanism requires that both Ca^{2+} dependent and independent adhesion co-exist and are responsible for the aggregation behavior of the cells. Chick embryonic cells will cross-adhere regardless of their tissue origin as long as they share one of these two classes of adhesion.

Hennings and Holbrook (3) and Hennings et al. (4) used epidermal cells from BALB/c mice to examine the divalent cation requirements of desmosome formation, a cell-cell adhesion junction. They observed asymmetric desmosomes when cells were cultured in low Ca^{2+} medium. 5 min after increasing the concentration of Ca^{2+} to 1.2 mM they found desmosomal plaques that had tonofilaments inserting into them, and after 2 h they observed symmetric desmosomes (desmosomal plaques opposite each other on opposing cells). By changing the concentration of Ca^{2+}, Jones et al. (5) demonstrated that the close association of intermediate filament bundles with desmosome formation in primary mouse epidermal cells was Ca^{2+} dependent. At low Ca^{2+} concentrations a bundle network of tonofilaments was located in the juxtanuclear region. After Ca^{2+} was added to the medium, the network moved toward the cell periphery and made contact with the cell membrane. Desmosome formation then increased dramatically.

In many organ and tissue systems control of the level of intracellular Ca^{2+} appears to be dependent on the ubiquitous Ca^{2+}-binding protein, calmodulin (CaM)[1] (6, 7). CaM controls a number of fundamental activities, such as cell proliferation and migration (8, 9) and Ca^{2+} transport (10).

It is not known whether divalent cations or CaM play a role in the maintenance and formation of the cell-substrate adhesion junctions such as hemidesmosomes (HDs). HDs are those adhesive junctions that attach basal cells of stratified squamous epithelia to their substrate, the basal lamina. In addition to providing a strong mechanical coupling, it is likely that these junctions, through their associated tonofilaments, exert tension and distribute the force throughout the cells, playing a role in the maintenance of cell shape.

[1] *Abbreviations used in this paper:* CaM, calmodulin; HD, hemidesmosome.

Except for the ultrastructural studies of Krawczyk and Wilgram (11) and of Beerens et al. (12), there has been little information available on HD formation. Recently, Gipson et al. (13) developed an in vitro system for studying HD formation. Intact sheets of rabbit corneal epithelium were placed on denuded basal laminae and incubated. Using this procedure, the investigators found that the majority of new HD formation occurred within the first 6 h of culture. By 24 h, >90% of the number of HDs per micron of membrane found in normal intact rabbit corneas had formed. As the length of culture time increased, the percentage of immature HDs decreased as the percentage of mature HDs increased. Immature HDs could be divided into two types. Type 1 was characterized by the presence of fine filaments between the membrane and the lamina densa, and Type 2 was characterized by the presence of an electron dense plaque on the cytoplasmic face of the membrane. Mature HDs (Type 3) were distinguished from immature HDs by the appearance of an extracellular electron dense line parallel to the membrane and the lamina densa. In addition, at this stage intermediate filaments that inserted into the electron dense plaque were often present. The major shift in HD maturation occurred during the first 6 h of culture. The investigators also observed that de novo HD formation occurred at sites on the basal lamina opposite existing anchoring fibrils. Anchoring fibrils insert into the lamina densa on the side opposite the basal cell plasmalemma and splay out among the collagen fibrils.

The in vitro system developed by Gipson et al. (13) provides a method for examining the role of divalent cations and CaM in HD formation. We found that HD formation is dependent on the concentration of Ca^{2+}; development of HD's into mature stages is Ca^{2+} dependent; epithelial basal cell shape is Ca^{2+} dependent and a change in cell shape from columnar to round decreases the extent of HD formation; and CaM antagonists reversibly inhibit HD formation.

MATERIALS AND METHODS

Animals and Tissues: Corneas from New Zealand white rabbits were used for all the experiments. A complete description of the removal of intact corneal epithelial sheets is found in Gipson and Grill (14) and the protocol for placing these epithelial sheets on basement membranes is explained by Gipson et al. (13). Briefly, a circular piece of cornea 9-mm diam was removed and placed in defined culture medium (15) containing 1.2 U/ml Dispase II (Boehringer Mannheim Laboratories, Inc., Indianapolis, IN), a bacterial neutral protease. The culture medium, Eagle's minimal essential medium with Earle's balanced salt solution, contained, per 100 ml, 2 mM glutamine, 0.1 mM nonessential amino acids, trace elements (0.46 μM $CoCl_2$, 0.28 μM $MnCl_2$, 0.1 μM $CuSO_4$, 0.17 μM $FeSO_4$, 0.05 μM $ZnSO_4$, 0.097 μM $(NH_4)_6\,MO_7\,O_{24}$), 100 U penicillin, 100 μg streptomycin, and 0.25 μg amphotericin B. The posterior half of the stroma was removed and the anterior half, with the attached epithelium, was then incubated for 1 h at 35°C in culture medium containing Dispase II. After 1 h, the epithelial sheet was teased off and placed on a smaller-diameter segment of cornea with denuded epithelial basal lamina. The epithelium-basal lamina combinations were then incubated in culture medium that either contained (*a*) varying concentrations of Ca^{2+} ions; (*b*) CaM antagonists; (*c*) CaM after an initial 6-h incubation with antagonists; or that lacked (*d*) Mg^{2+} ions. The time periods chosen to examine HD formation were 3, 6, 18, or 24 h.

Ca^{2+} Ion Concentration in Media: HD formation was determined after incubation in varying concentrations of Ca^{2+}. To prepare the different concentrations, $CaCl_2$ or EGTA was added to the low Ca^{2+} medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) and seven concentrations were prepared: 0.5, 5, and 10 μM; 0.1, 0.3, 0.6, and 1 mM Ca^{2+}. The low Ca^{2+} medium contained the same additives as the control-defined culture media described above. Control medium contained 1 mM Ca^{2+}, whereas low Ca^{2+} medium contained 10 μM Ca^{2+}. 0.5 and 2.0 mM EGTA concentrations were used to produce the final Ca^{2+} concentrations of 5.0 and 0.5 μM. The medium was buffered to pH 7.4 with monobasic sodium phosphate buffer. HD formation in low concentrations of Ca^{2+} ions was compared with the data obtained from that in 1 mM Ca^{2+} (13).

Determination of Free and Bound Ca^{2+} Concentrations: The concentration of free Ca^{2+} was determined with a Ca^{2+} ion selective electrode. For each solution, the concentration of free Ca^{2+} in the medium was tested before incubating the tissue and again after a 6-h incubation period. The tissue was taken after incubation in medium containing varying concentrations of Ca^{2+}, and the total Ca^{2+} in the cornea was determined by atomic absorption spectrophotometry.

Use of CaM Antagonists and CaM in Culture: To determine whether HD formation was CaM dependent, epithelial-basal lamina combinations were incubated for 6 h in medium containing 1 mM Ca^{2+} and 40 μM W7 or W5, two CaM antagonists. The antagonists were initially dissolved in dimethyl sulfoxide and then diluted with culture medium. In half the experiments the medium was changed at 6 h and the tissue was incubated for an additional 12 h in the absence of the antagonists. To further examine the effect of CaM on HD formation, the epithelial-basal lamina preparations were incubated for 6 h in the presence of the antagonist W7, and after 6 h culture, corneas were washed in three changes of defined medium and cultured for an additional 12 h in the same medium containing 2 μM CaM.

[^{3}H]Leucine Incorporation of Corneal Epithelium: To determine if low Ca^{2+} culture conditions or the presence of CaM antagonist (W7) affected the metabolic activity of epithelial-basal lamina preparations, activity was measured by [^{3}H]leucine incorporation into trichloroacetic acid-precipitable material. The medium contained 10 μM Ca^{2+}, 1 mM Ca^{2+}, or 1 mM Ca^{2+} with 40 μM W7. The corneas were incubated for 6 h total and for the last 3 h [^{3}H]leucine was present at a concentration of 2 μCi/ml. After 6 h the epithelial sheets were removed by scraping (16). Samples were frozen in liquid N_2 and transferred to precooled 1-ml disposable culture tubes (Kimble, Div., Owens-Illinois, Inc., Toledo, OH). After harvesting, 250 μl water was added to each sample, and the stoppered tubes were placed in a Branson B-3 Sonic Cleaner for 5 min to disperse the tissue. An equal volume of 15% (wt/vol) trichloracetic acid was then added, and the tubes were vortexed, stoppered, and allowed to precipitate overnight at 4°C. After 15 min of centrifugation (1,600 *g*), the supernatants were drawn off and the pellets were washed three times with 200 μl 7.5% (wt/vol) trichloracetic acid with centrifugation. The pellets were solubilized into 200 μl of 0.2 M NaOH by incubating the stoppered tubes at 80°C for 2 h. Protein was determined by the Bradford (17) dye-binding method (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as the standard. The neutralized aliquots were counted for radioactivity in Aquasol-2 (New England Nuclear, Boston, MA) using a Beckman LS-8100 (Beckman Instruments, Inc., Palo Alto, CA). For comparison, all results were determined as disintegrations per minute per milligram of protein.

Tissue Processing for Electron Microscopy: After incubation, the tissue was fixed for 1 h in 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), and postfixed in 2% OsO_4 in cacodylate buffer (pH 7.4) for 1 h. *En bloc* staining with 0.5% uranyl acetate was followed by tissue dehydration in a graded series of acetone and of propylene oxide. Tissue was embedded in Epon-araldite for light and electron microscopy. 1-μm sections were stained with toluidine blue, and from a region with columnar basal cells 0.6-μm sections were cut for electron microscopy. Light micrographs were taken on a Zeiss photoscope III and electron micrographs on a Philips EM 200.

Statistical Analysis: A minimum of four experiments were conducted for each treatment. Electron micrographs were taken of basal cell membranes of eight cells for each experiment and the total number of HDs were counted by two independent investigators. The mean number of HDs per micron of membrane was recorded. The type of HD (immature or mature) was recorded according to the designation of Gipson et al. (13). All data were presented in the form of the mean ± SEM. Mann Whitney U tests were conducted to determine whether or not the number of HDs present for one treatment differed significantly from those receiving another treatment. The density of HDs per micron of membrane formed in low Ca^{2+} containing media was compared with that found in control medium.

RESULTS

Effect of Ca^{2+} on HD Formation

The number of HDs that had formed on basal cells of corneal epithelium after incubation on basal lamina in medium containing low Ca^{2+} (10 μM) for 3, 6, 18, or 24 h is shown in Fig. 1. The upper line (×) denotes formation in the control medium containing 1 mM Ca^{2+} (13) and the lower line (○) represents formation in the low Ca^{2+} medium. The

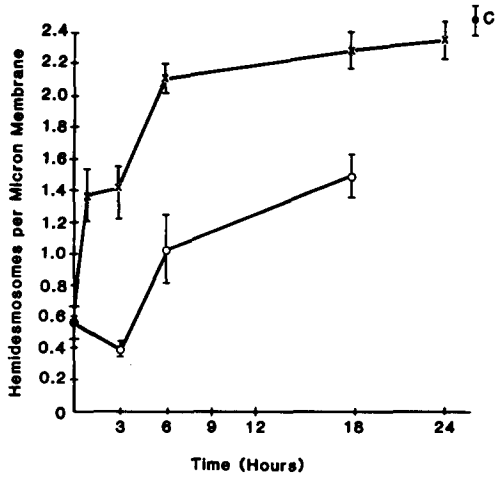

FIGURE 1 The number of HDs per micron of membrane in defined medium containing 1 mM Ca^{2+} (×) or 10 μM Ca^{2+} (○) is illustrated. HDs were counted along basal membranes for eight cells per experiment. A minimum of four experiments were conducted. The number of HDs present in control cornea is indicated by C. The zero point indicates the number of HDs present immediately after an intact corneal epithelial sheet is removed.

mean of the remnant HD plaques present on epithelial sheets immediately after removal and without any incubation was represented as the zero time point. The number of HDs formed in the low Ca^{2+} media was less than the control at 3, 6, and 18 h. However, the rate of formation did not differ significantly for the two concentrations of Ca^{2+} between 3 and 6 h (Mann Whitney U test, $p \leq 0.05$). After 6 h, the rate of formation decreased more sharply in the control medium than in the medium containing a lower concentration of Ca^{2+}. Formation from 6 to 18 h in 10 μM Ca^{2+} was more than twice that in 1 mM Ca^{2+}. During this time period not only did HD formation continue but also the number of mature HDs increased. After 18 h in low Ca^{2+} medium epithelial cells became edematous, and a 24 h time point could not be determined because of the deleterious effects of prolonged culture on epithelium in low Ca^{2+} medium.

To determine if HD formation was correlated to changes in Ca^{2+} concentration, the number of HDs formed per micron of membrane in the presence of five Ca^{2+} concentrations was determined. At both 6 and 18 h of incubation time, the number of HDs increased with increasing concentration of Ca^{2+} (Fig. 2). The number of HDs formed after 6 h did not differ significantly for the three lowest Ca^{2+} concentrations (Figs. 2 and 3, *a* and *b*). However, a significant increase in HDs occurred when culture medium contained 0.6 mM Ca^{2+}. A similar increase occurred when medium contained 1.0 mM Ca^{2+}. The number of HDs present after 18 h in six concentrations of Ca^{2+} ranging from 5 μM to 1 mM Ca^{2+} was observed to follow a gradual step-like transition (Fig. 2). The greatest increase in the density of HDs occurred between 0.3 and 1.0 mM Ca^{2+}. The increase in the number of HDs with increasing Ca^{2+} concentration can be seen in the electron micrographs in Fig. 3. In low Ca^{2+} medium (Fig. 3*a*) only a small number of mature HDs were present and these were distributed sporadically along the basal lamina. From Fig. 3, *b* and *d*, the number of HDs increased with the corresponding higher concentrations of Ca^{2+}. At 1.0 mM Ca^{2+} (Fig. 3*d*) HDs were prominent along the basal membrane and were also associated with the intermediate filaments. The distribution of HDs along the cell membrane was more regular at higher Ca^{2+} concentrations (Fig. 3*d*).

HD formation did not require Mg^{2+} ions. In medium lacking Mg^{2+}, the HDs per micron of membrane were 2.1 ± .27 after 6 h as compared with 2.1 ± .08 in control medium. The percentage of mature HDs did not differ significantly from that found when incubated in the control medium (Mann Whitney U test, $p < 0.02$).

Effect of Ca^{2+} Concentration on HD Maturation

Maturation of HDs depended on both Ca^{2+} concentration and length of incubation. Table I shows the percentage of mature HDs at 3, 6, and 18 h in varying Ca^{2+} concentrations. At 18 h the greatest percentage of mature HDs was found in control medium, and then decreased with the decreasing Ca^{2+} concentration. The percentage was greater at 10 μM than at 0.1 mM; however, the difference was not significant. Although the percentage of mature HDs was lower at 6 h for all concentrations, the same trend was apparent (Table I). At 3 h, mature HDs were present only at 1 mM Ca^{2+}. Although formation occurred when incubated in medium containing <10 μM Ca^{2+}, only immature stages were present. The number or maturity of HDs per micron of membrane did not affect their association with anchoring fibrils located beneath the lamina densa of the basement membrane. More than 90% of the HDs present were associated with anchoring fibrils (Fig. 3). A high association of the HDs present to the underlying fibrils agreed with the data of Gipson et al. (13).

Effect of CaM Inhibitors on HD Formation

Phenothiazines, such as trifluoperazine (TFP), and naphthalenesulfonamides, such as the W series (W7 and W5), bind to CaM in a Ca^{2+}-dependent manner and inhibit Ca^{2+}-CaM regulated activities (9, 18–22). Two specific antagonists, W5 and W7, were added to the culture medium to test their effect on HD formation. HD formation was negligible when 40 μM of W7 was added to the control medium and incubated for either 6 or 18 h (Table II). Only immature HDs were present. The number of HDs per micron of membrane was 10.7% of the control at 6 h and 9.6% of the control at 18 h. When the antagonist was removed after 6 h incubation, the corneas rinsed and incubated for an additional 12 h in control medium, formation occurred and was significantly greater than

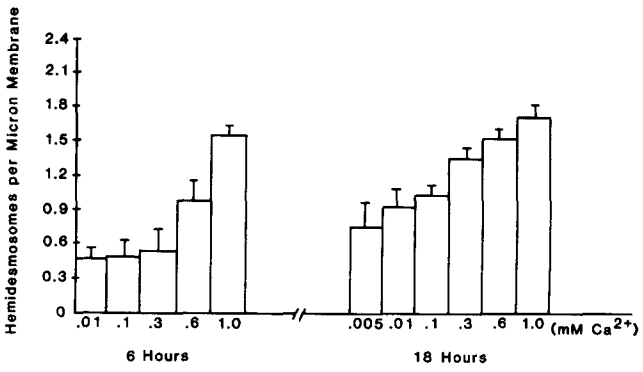

FIGURE 2 HD formation per micron of membrane is depicted at varying concentrations of Ca^{2+} in defined medium after 6 and 18 h. Numbers are calculated as the number of HDs present at 0 h subtracted from the number present at 6 or 18 h.

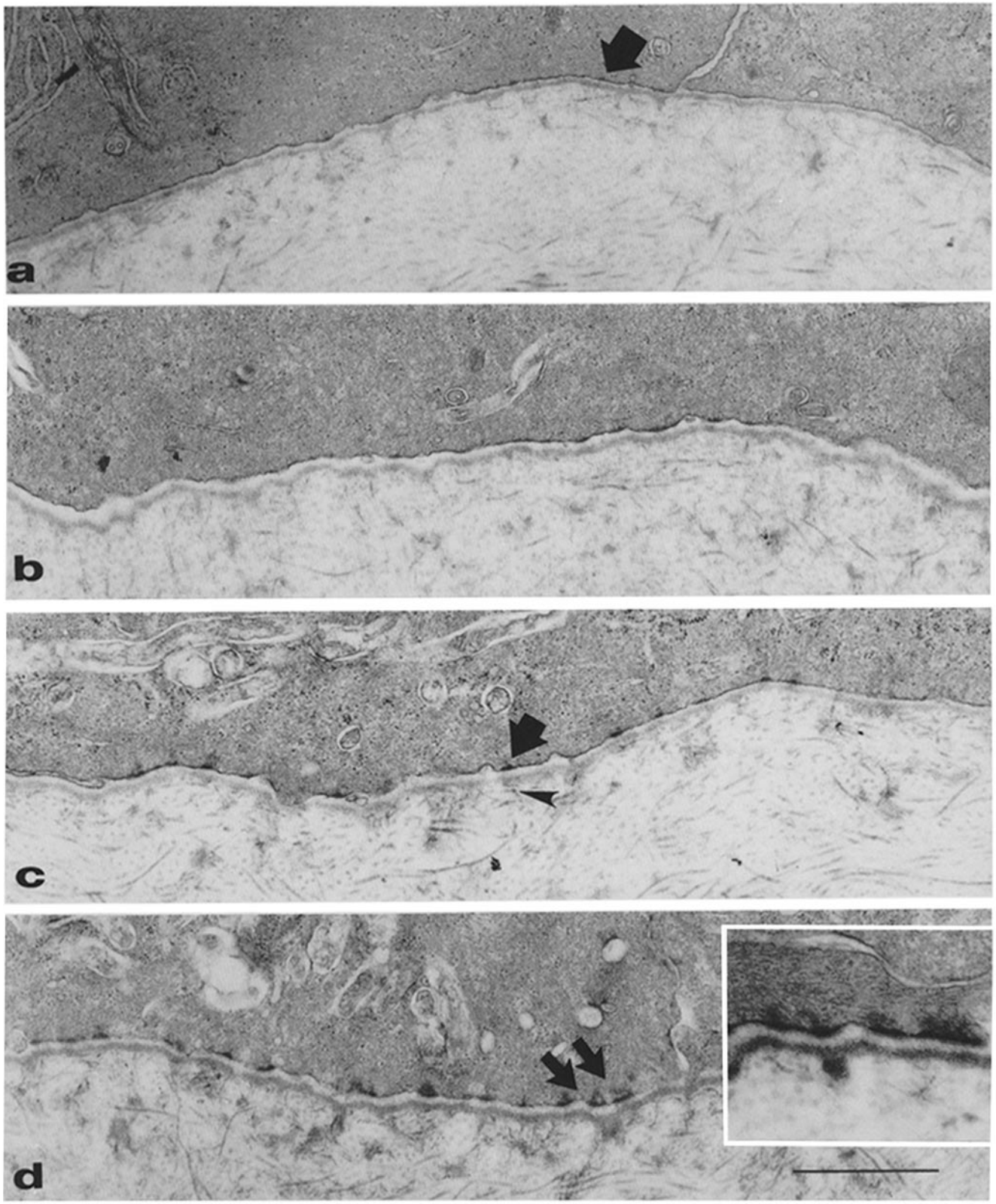

FIGURE 3 Electron micrographs of epithelial-basement membrane preparations show HD formation along the basal membrane. Formation is shown in the four concentrations of Ca^{2+} after 6 h of incubation. Single arrows point to hemidesmosomes. Double arrows point to tonofilaments and the single arrowhead points to anchoring fibrils. Inset shows insertion of tonofilaments. (*a*) 0.01 mM Ca^{2+}; (*b*) 0.3 mM Ca^{2+}; (c) 0.6 mM Ca^{2+}; (*d*) 1.0 mM Ca^{2+}; Bar, 1.0 μm. × 25,000. (*inset*) × 50,000

formation in the presence of W7 (Mann Whitney U test, $p \leq 0.001$). Although the number of HDs did increase, the number per micron of membrane was only 57% of the control. The extent of formation and the percentage of mature HDs (20%) resembled that in cultures containing 10 μM Ca^{2+}. When 2 μM of CaM was added to the control medium for the second half of the incubation, the number of HDs per micron of membrane was significantly higher than that attained with

TABLE I

*Percentage of Mature Hemidesmosomes**

Ca^{2+} concentration	% HD at three time intervals		
	3 h	6 h	18 h
mM			
Control (1.0)	19.0 ± 3.7	27.5 ± 2.9	31.8 ± 4.0
0.3		9.5 ± 2.7	26.5 ± 3.8
0.1		8.8 ± 2.7	16.3 ± 5.6
0.01		7.3 ± 3.1	18.1 ± 4.7
0.005		0	0
0.0005	0	0	0

*Type 3 (13).

TABLE II

Effect of CaM Antagonist on Hemidesmosome Formation per Micron of Membrane

Time	Control	W7	W7 → CaM
h			
6	2.13 ± .08	0.23 ± .03*	
12	2.20‡		
18	2.29 ± .12	0.22 ± .05	
6 → 12 wash		1.27 ± .23	1.58 ± .29

* SEM, n greater than four experiments; eight cells per experiment.
‡ Extrapolated from Fig. 1.

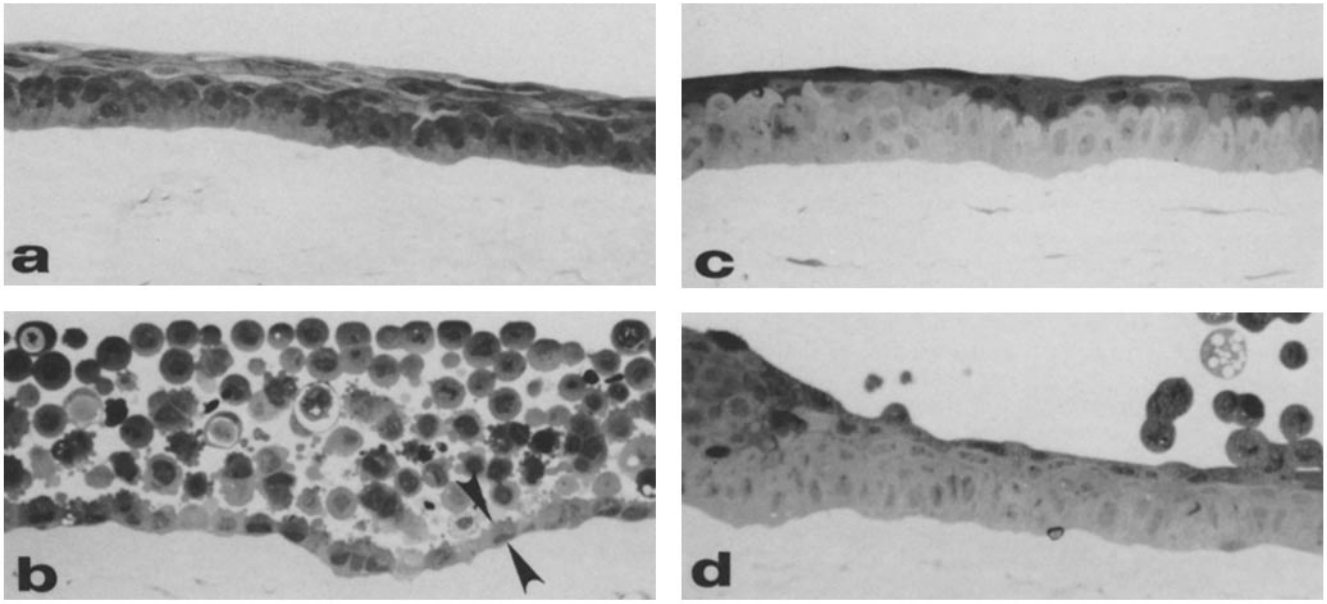

FIGURE 4 Photomicrographs of 1-μM sections of epithelial-basement membrane preparations, illustrating an intact layer of epithelial basal cells (two arrowheads) after incubation in defined medium for 6 h. Four different treatments are shown: (*a*) 1 mM Ca^{2+}; (*b*) 0.01 mM Ca^{2+}; (*c*) no Mg^{2+}, (*d*) 1 mM Ca^{2+}; and 40 μm W7. × 300.

the addition of control medium alone (Mann Whitney U test, $p \leq 0.05$). 72% of the control number of HDs per micron of membrane was present after 12 h. Formation did not occur when 2 μM of CaM was added to medium containing 10 μM Ca^{2+} and 2 mM EGTA. W5, a control for nonspecific effects of W7 (9, 22) did not inhibit HD formation as extensively as did W7. 30% more HDs were present along the basal membrane when W5 instead of W7 was added to the control medium.

Influence of Low Ca^{2+} and CaM Antagonists on Protein Synthesis

Culture in low Ca^{2+} containing medium and control medium containing 40 μM W7 did not significantly affect the metabolic state in any of the three media examined (10 μM Ca^{2+}, 1 mM Ca^{2+} [control], and 1 mM Ca^{2+} with 40 μM W7). After a 6-h culture, [^{3}H]leucine incorporation into trichloracetic acid-precipitable proteins was 99% and 94.8% of the control, respectively. Thus the lower number of HDs per micron of membrane present after treatment with W7 does not appear to be the result of depressed metabolic activity.

Cell Architecture and Cell-Cell Adhesion Organelles

When incubated for 18 h, epithelial sheet architecture and cell shape were influenced by the concentration of Ca^{2+}. At concentrations >0.3 mM Ca^{2+}, or in Mg^{2+}-free medium, the epithelial sheets on the basement membranes displayed normal continuous apical-basal stratification with columnar basal cells (Fig. 4). At lower Ca^{2+} concentrations or in the presence of CaM antagonists, basal cells spread along the basal lamina and stratification was focal. The lack of cell shape maintenance at the lowest Ca^{2+} concentrations was even seen at 3 h. Although neither HDs nor desmosomes were present at the lowest Ca^{2+} concentrations, one layer of cells constantly adhered to the basal lamina (Fig. 4*b*).

DISCUSSION

We have determined that HD formation, which occurs when freed sheets of rabbit corneal epithelium are placed on denuded corneal basal laminae, requires Ca^{2+} and is mediated by CaM. The number of HDs formed is dependent on the

concentration of Ca^{2+} and HD maturation is a reflection not only of time but also of Ca^{2+} concentration. In addition we have found that the shape of the epithelial basal cells affects normal HD formation and that the CaM antagonist, W7, inhibits HD formation in a reversible manner.

The ionic requirements for HD formation, a cell-substrate adhesion junction, resemble those of cell-cell adhesion junctions, desmosomes. Hennings et al. (4) and Hennings and Holbrook (3) showed that desmosomal formation between mouse epidermal cells required Ca^{2+}. They established this requirement by showing the absence of formation in low Ca^{2+} medium and the return of formation 2 h after the concentration of Ca^{2+} was restored.

After 6 h of culture, HD formation in low Ca^{2+} medium occurred at a faster rate than that in control medium. This contrasts to the extent of formation, which is lower than that observed in control medium. These observations may be explained by the hypothesis that the number of HDs that form is controlled by the number of available sites. Using the in vitro system, Gipson et al. (13) presented data that indicate that HDs form over sites on the basal lamina where anchoring fibrils insert. They also demonstrated that >80% of the number of HDs present in control corneas had formed by 6 h. Thus by 6 h in control medium most of the available sites had been filled. After 6 h in control medium the rate of formation leveled off because only a small number of available sites remained. In low Ca^{2+} medium, since the extent of formation is lower, we hypothesize the rate of formation to be higher after 6 h because many sites are available. It is possible that the mobilization of intracellular Ca^{2+} during incubation might permit the higher rate depicted in the low Ca^{2+} medium.

Our data indicate that HD maturation not only is time dependent (13) but also is Ca^{2+}-concentration dependent. The smaller percentage of mature HDs that possess intermediate filaments inserting into their plaques in low Ca^{2+} medium may be related to the observation that Ca^{2+} is required for intermediate filaments to associate with adhesion plaques. Jones et al. (5) recently described the behavior of intermediate filament bundles in low Ca^{2+} medium in primary mouse epidermal cells and observed that the intermediate filament bundles were generally located in the juxtanuclear region of the cell. They also reported that intermediate filaments rearrange, move to the cell periphery, and make contact with desmosomes after the addition of Ca^{2+}. Our data support these observations and indicate that the association of intermediate filaments to HDs is Ca^{2+} dependent.

Since CaM is known to regulate a number of fundamental activities such as glycogen metabolism, intracellular motility, Ca^{2+} uptake, and DNA synthesis (6, 7, 10, 23–25), the role of CaM in the formation of cell-substrate adhesion junctions is not surprising. The results indicate that even though the excess antagonist is removed and fresh medium added for the second half of the incubation, some of the CaM present in the cell remains bound to the antagonist in the tissue. Therefore, both CaM and Ca^{2+} are required for further formation. The CaM that is added to the medium may either cause the antagonist to disassociate from the CaM in the cell and act as a sink or it may enter the cell and provide binding sites that have been taken by the binding of the antagonist to CaM. Entrance of CaM into the cell may be possible as the membranes are presumably altered after incubation with antagonist (26). Studies using radioactively labeled exogenous CaM need to be conducted before one can ascertain whether or not CaM enters the cell. When CaM was added to medium that contains 10 μM Ca^{2+} and 2 mM EGTA, no HD formation occurred, because the EGTA competes with CaM for free Ca^{2+} thus removing it from the system. Our data indicate that HD formation is a Ca^{2+}-CaM regulated activity.

The shape of the epithelial basal cell appears to be of prime importance in HD formation as HDs with the greatest density formed when basal cells of the epithelial sheet were columnar and when the ionic requirement for Ca^{2+} was met. Jones et al. (5) showed that the intermediate filament bundle system of desmosomes (cell-cell junctions) is important in cell shape maintenance. After 1 h in low Ca^{2+} medium, desmosomes were not able to maintain their structural integrity as the intermediate filament bundles moved from the cell periphery, and the cell deviated from its "native" columnar shape. Once Ca^{2+} was returned to the medium and the desmosomes reformed (within 30 min), the cells began to acquire a more columnar shape. Our experiments agree with the findings of Jones et al. (5) and Hennings et al. (4) that desmosome formation occurs soon after the concentration of Ca^{2+} in the medium is restored. However, HD formation does not occur as rapidly. It was not until 6 h of incubation in control medium after the low Ca^{2+} incubation that any HD formation occurred. Formation was observed only at sites where several adjacent cells had returned to their columnar shape.

Pitelka et al. (27) have also observed contortion of mammary epithelium grown on collagen when chelators such as EGTA or sodium citrate are added. Even though cell-substrate adhesion is maintained, the distortion of these cells may be attributed to the centripetal tension within each cell. When low Ca^{2+}-containing medium was used in our organ culture system, the basal cell layer of the corneal epithelium adhered to the basal lamina and the characteristic hump shape of the cells was present as described by Pitelka et al. (27). The increase in the concentration of Ca^{2+} caused the corneal epithelial cells to become more columnar in shape. HD formation appears to require the precise alignment of basal cells and the initial adherence to the basement membrane. Only after cell substrate alignment and adhesion did assembly, synthesis, and maturation of HDs occur. The importance of the epithelial basal cell shape and its realignment is supported by the work of several investigators. Following subepidermal blister induction, Beerens et al. (12) observed that the initial step of HD formation was the realignment of the basal cells to the basement membrane. They suggested that the HD remnants from the operation were phagocytosed before formation. Krawczyk and Wilgram (11) noted that mature HDs were present only beneath fully attached nonmigrating keratinocytes. This observation was supported by that of Buck (28), who showed that there was only a patchy distribution of HDs near the migrating marginal cells of healing mouse corneal epithelium. HD number was suppressed as far away as 1.5 mm from the migrating cells.

The molecular mechanisms involved in the regulation of HD formation remain to be elucidated. Our results indicate that formation and maturation is a multi-step mechanism that uses both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms. Dual adhesion systems have been reported by several investigators (1, 2, 29) in their cell-cell molecular adhesion systems in the developing chick embryo. In the present system, initial adhesion of the corneal epithelial basal cell layer occurred at the lowest concentrations of Ca^{2+}. Second, for-

mation of HDs was inhibited by CaM antagonists in a reversible manner. In low concentrations of Ca^{2+}, most of the cell's energy seems to be directed toward formation and not maturation, as indicated by the longer periods of high rates of formation. Third, maturation may be influenced by the mobilization of the intermediate filament bundles to the cell periphery. Our culture system allows the study of different phases of HD formation under controlled ionic conditions. Utilization of the model system of Gipson et al. (13) may facilitate the understanding of the regulation of HD formation and the role of Ca^{2+} and CaM in controlling cell-substrate interactions.

We thank R. Mikkelson of Tufts University for the use of his Ca^{2+}-sensitive electrode and M. Donawitz of Tufts University for running samples on the atomic absorption spectrophotometer. In addition, we thank Sandra J. Spurr, Steven Brennan, and Ann Tisdale for technical assistance.

This research was supported by a National Institutes of Health (NIH) grant R01 EY03306 to I. K. Gipson, and postdoctoral fellowships from NIH (F32 EY05614) and Fight for Sight, Inc., New York City supported V. Trinkaus-Randall.

Received for publication 11 August 1983, and in revised form 5 December 1983.

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