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Protein Kinase A and Protein Kinase C Modulators Have Reciprocal Effects on Mesenchymal Condensation during Skin Appendage Morphogenesis

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The molecular signaling of secondary induction is a fundamental process in organogenesis during embryonic development. To study the signal transduction pathways involved, we used developing chicken skin as a model and focused on the roles of intracellular signaling during feather morphogenesis. Protein kinase C (PKC) immunoreactivity increases in the whole layer of forming dermis around H and H stage 30. This is followed by a gradual and highly localized decrease of PKC expression immediately beneath each forming feather germ. In contrast, cAMP response element binding protein (CREB) is ubiquitously expressed in both epithelium and mesenchyme. From stage 29 on, phosphorylated CREB (P-CREB), reflecting the activity of protein kinase A (PKA), begins to be seen in placode but not in interplacode epithelia. P-CREB is also expressed in bud mesenchyme transiently between stages 33 and 36, but not in the interbud mesenchyme. The presence and activity of PKC, PKA, and P-CREB in developing chicken skin are further characterized by immunoblot, kinase activity, and gel shift assays. To explore their physiological significance, embryonic chicken dorsal skin explants were treated with different modulators in medium or in beads for localized effects. The results showed that PKA activators and PKC inhibitors can expand a feather bud domain by enhancing dermal condensation, while PKC activators and PKA inhibitors can expand interbud domains. Neural cell adhesion molecule (N-CAM) is involved in dermal condensation. We observed that activation of PKA causes diffused expression of N-CAM in mesenchyme while activation of PKC causes the disappearance of N-CAM in precondensed mesenchymal regions. A model of how the well-concerted PKA and PKC signaling may be involved in the formation and size regulation of dermal condensation is presented.

INTRODUCTION

Induction is one of the most fundamental processes in developmental biology. During induction two groups of cells interact and produce altered morphogenetic or differentiation fates (Jessel and Melton, 1992; Slack, 1993). In recent years remarkable progress was made on Xenopus on primary induction that set up the basic body plan in early embryos. Several growth factors such as TGF-β, FGF, activin, Wnt, and bone morphogenic protein are involved (for reviews, see Melton, 1991; New *et al.*, 1991; Slack, 1993). Intracellularly, signaling molecules such as protein kinase C (PKC) (Otte *et al.*, 1988, 1991; Otte and Moon, 1992) and protein kinase A (PKA) (Otte *et al.*, 1989) are shown to be involved in Xenopus neural induction, and ras is shown to be involved in mesoderm induction (Whitman and Melton, 1992). In contrast, many fewer studies of molecular events underlying secondary inductions, which occur between different germ layers in later embryos and result in organogenesis, have been done.

We are interested in studying molecular signals involved in the secondary induction (classical phenomena were summarized in Sawyer and Fallon, 1983). The feather, with its repetitive pattern and accessibility to experimental manipulation, is one of the best models. Classical studies have established the importance of epithelial–mesenchymal interactions (reviewed in Sengel, 1976) which lead to a series of morphogenetic events from a flat piece of epithelium and mesenchyme through the formation of feather placodes,

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dermal condensations, protrusion of feather buds, growth of feather buds, and invagination of feather follicles (reviewed in Chuong, 1993). Despite some recent understanding of the molecules involved in the formation of skin appendages including hair, feathers, and scales (for review, see Hardy, 1992; Chuong *et al.*, 1991, 1993; Kaplan and Holbrook, 1994), information on signals involved in inductive events is lacking. For example, we do not know what signals cause the initial formation of feather placode and what signals are exchanged between epidermis and mesenchyme in these critical periods. This laboratory has recently shown that retinoic acid (RA) can cause the forming dermal condensations to disappear (Chuong *et al.*, 1992) and that neural cell adhesion molecule (N-CAM) is involved in mediating dermal condensation (Jiang and Chuong, 1992), but we still do not know what signals initiate the molecular cascade of dermal condensations. By making an analogy with primary induction, it is likely that some growth factors will be involved in secondary induction as well. Recently, growth factors such as the TGF-β superfamily have been shown to mediate alterations in dental mesenchyme in secondary induction (Vainio *et al.*, 1993). Similar to the case of mesoderm induction in Xenopus, the effect of growth factors in secondary induction appears to be redundant and cross-reactive.

While trying to elucidate the effect of extracellular signaling molecules such as TGF-β on the induction of feather buds (Ting-Berreth and Chuong, submitted for publication), we reasoned that the effects of the extracellular signaling molecules have to be integrated at the cell membrane and translated into intracellular signals. A study focusing on the changes in intracellular signaling in induction could provide a more unified picture and enhance our efforts to search for upstream regulators and downstream targets. Thus we set out to find intracellular signal transduction pathways which are involved in the events of skin appendage induction. We tested different molecules which participate in intracellular signaling pathways, among which were phosphorylated cAMP response element binding protein (P-CREB) and PKC, which are involved in cAMP and diacylglycerol (DAG) signaling pathways. Unexpectedly we found that P-CREB and PKC are expressed complementarily in feather bud and interbud regions, respectively. This finding prompted us to hypothesize that P-CREB has a role in the formation of the feather bud domain while PKC is involved in the formation of interbud domains. Tests on chicken dorsal skin using biochemical assays and pharmacological modulators supported this hypothesis. In addition, expression of N-CAM, which mediates dermal condensation, was altered by modulating the above signaling pathways. A model showing the reciprocal activity of P-CREB and PKC in the formation of dermal condensations and intercondensation mesenchyme is presented.

MATERIALS AND METHODS

Embryos

White leghorn fertilized chicken eggs were obtained from K and R Farm (Westminster, CA) and Chino Valley ranchers (Chino, CA). Eggs were incubated at 37°C and embryos were staged according to Hamburger and Hamilton (1951).

Antibodies

Antibodies to PKC are from Upstate Biotechnology Incorporated (UBI; Lake Placid, NY). This is a rabbit polyclonal antibody raised against a synthetic peptide of the 40 C-terminal amino acid of PKC β II conjugated to keyhole limpet hemocyanin (KLH) (Hagiwara *et al.*, 1990). The PKC family is composed of more than 12 isozymes, including the classical (α, β, r) and nonclassical type (Huang, 1989; Stabel and Parker, 1991; Dekker and Parker, 1994). This PKC antibody from UBI was stated to be a pan-PKC antibody against all of the classical isoforms. We chose a PKC antibody with wider specificity because it is an initial study. Future studies will involve different isoforms.

Antibodies to CREB and P-CREB are also from UBI. CREB was made against the N-terminal 205-amino-acid fusion protein. P-CREB was made against a synthetic peptide (equivalent to amino acid 123–136) which was phosphorylated by a cAMP-dependent protein kinase and then coupled to KLH (Ginty *et al.*, 1993). Antibodies to N-CAM are described in Jiang and Chuong (1992).

Immunochemical Staining: Whole Mount and Sections

Whole-mount staining was done according to Klymkowsky and Hanken (1991). Briefly, skin was fixed in 20%

TABLE 1

Pharmacological Reagents Used for the Skin Explant Cultures

Reagent	Supplier	Stock concentration	Solvent
dbcGMP	Sigma	100 m*M*	DMEM
dbcAMP	Boehringer	100 m*M*	DMEM
8(4CPT) cAMP	Boehringer	100 m*M*	DMEM
Forskolin	Sigma	10 m*M*	Ethanol
H8	Seika Gaku	20 m*M*	DMEM
Ro20-1724	Gibco BRL	2 m*M*	0.7% DMSO
Calphostin C[a]	Calbiochem	20 μM	Ethanol
B-PDiac.	Sigma	1 m*M*	DMSO
PMA	Sigma	1 m*M*	Ethanol

Note. Abbreviations used: B-PDiac., 4β-phorbol-12,13-diacetate; 8(4CPT) cAMP, 8-(4-chlorophenylthio)adenosine-3':5'-cyclic monophosphate; dbcAMP, dibutyryl adenosine-3':5'-cyclic monophosphate; dbcGMP, dibutyryl guanosine-3':5'-cyclic monophosphate; H8, N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; PMA, phorbol 12-myristate 13-acetate.

[a] Addition of calphostin was followed by 1 hr of light illumination.

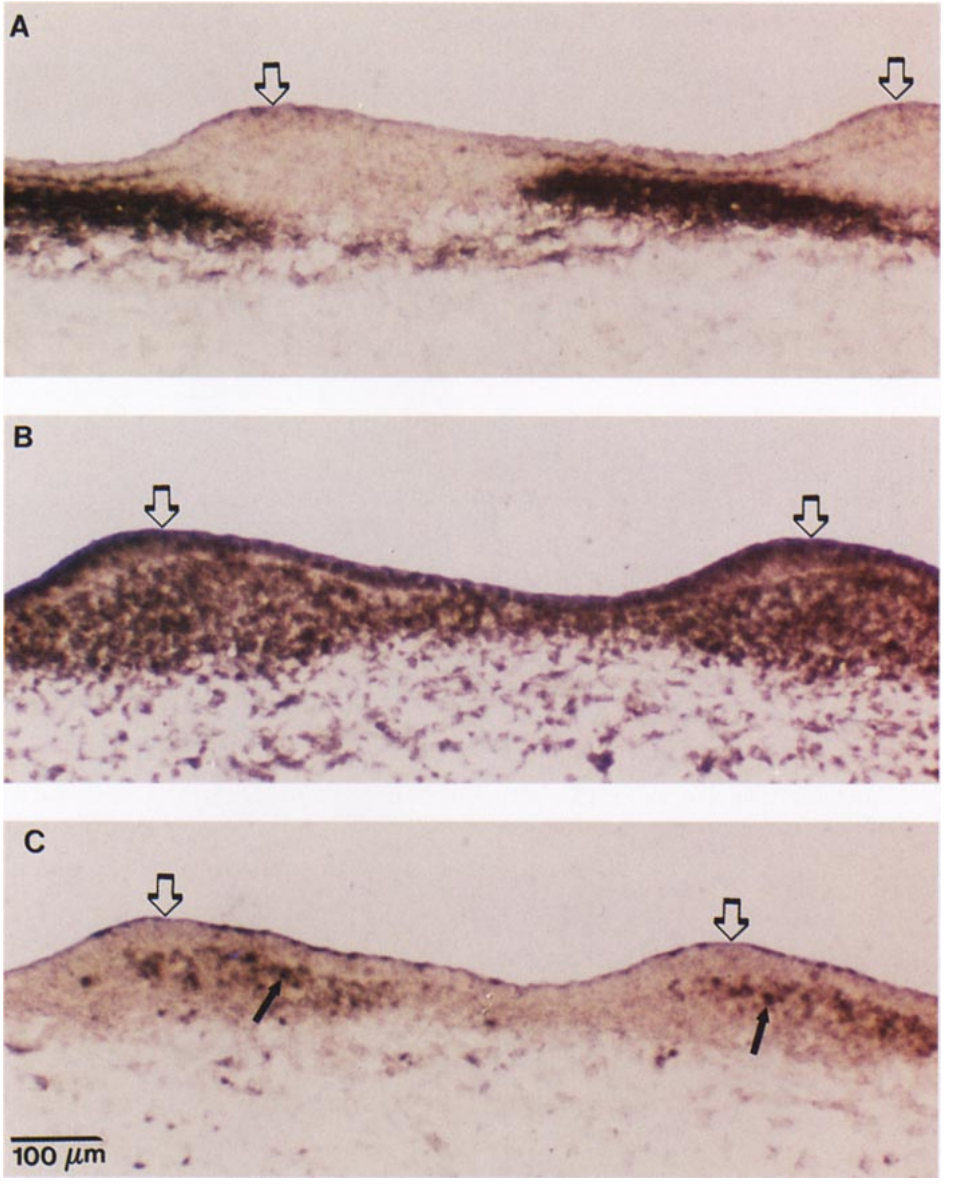


FIG. 1. Distinct expression patterns of PKC, CREB, and P-CREB in embryonic skin. Immunoalkaline phosphatase staining on paraffin sections of stage 32 (E7.5) embryonic chicken dorsal skin. Sagittal sections. Anterior (Anterior of the feather bud is defined by the side where the future rachis will form (Chuong *et al.*, 1990)) is to the right. Brown color is positive. (A) PKC. (B) CREB. (C) P-CREB. Note the entirely different distribution pattern. PKC is enriched in the interbud germ region. CREB is ubiquitously present in epidermis and dermis. P-CREB is enriched in feather germ. Open arrow, feather germ. Small arrow, nuclear staining pattern of P-CREB.

DMSO/80% methanol and bleached in 10% hydrogen peroxide diluted in the above solution for 1–2 days. Endogenous peroxidase activity was inhibited during bleaching. The specimen was then washed in Tris-buffered saline (TBS) and incubated overnight with 10 μg/ml anti-NCAM primary antibodies diluted in 95% calf serum/5% DMSO. The next day, specimen was washed in TBS 5 times for 1 hr each. This was followed by reaction with biotin-conjugated secondary antibodies under similar conditions as the primary antibody. Finally, avidin–peroxidase was added and color was developed. Following the enzyme reaction, tissue was cleared in benzyl benzoate:benzyl alcohol, mounted in a slide chamber, and observed under a microscope.

Paraffin sections using alkaline phosphatase-conjugated secondary antibodies were used as described in Chuong *et al.* (1990). Immunofluorescence on frozen sections was performed as described in Jiang and Chuong (1992).

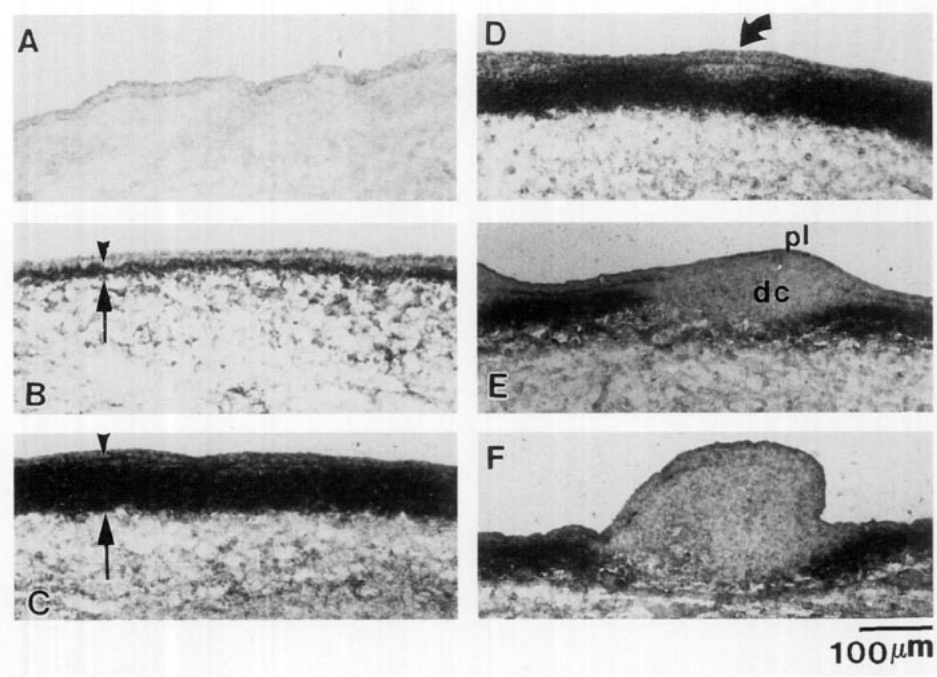


FIG. 2. Temporal and spatial expression of PKC during feather bud formation. Sagittal sections of dorsal skin. Anterior is to the left. Immunoalkaline phosphatase staining. (A) Stage 29 (E6) skin does not express PKC. (B) By stage 30 (E6.5) PKC starts to appear in superficial layer of dermis close to epidermis. (C) At stage 31 (E7) PKC is expressed ubiquitously throughout dermis. (D) At stage 32 (E7.5) PKC starts to disappear from the dermis of the growing feather bud but remains in the interbud dermis. (E) At stage 34 (E8), disappearance of PKC from feather bud dermis continues. (F) By stage 35 (E9) PKC has mostly disappeared from feather bud dermis and remains only in interbud dermis. dc, dermal condensation; pl, placode. Arrow and arrowhead point to the regions that are PKC-positive. The thick oblique arrow shows the initiation site of feather germ where PKC begins to disappear. Size bar, 100 μm.

Serum-Free Skin Explant Cultures

Embryonic chicken skin explants were cultured as described in Jiang and Chuong (1992). Dulbecco's modified Eagle's medium (DMEM) without serum was used. Pharmacological reagents were directly added to the medium and cultures were incubated for 3–6 days at 37°C.

Pharmacological Reagents

The reagents and vehicles are listed in Table 1. For local delivery, AG1-X8 anion exchange beads of 100–200 μm diameter (Bio-Rad) were soaked for 0.5–1 hr in 100 m*M* solution of 8-(4-chloro-phenylthio)-adenosine-3′:5′-cyclic monophosphate (8(4CPT)cAMP) (Miller *et al.*, 1975) or DMEM (control). After incubation the beads were washed with 1 ml of DMEM and centrifuged for 10 sec in a Picofuge (Stratagene). Washing and centrifugation were repeated two more times before use.

Immunoblot

Immunoblot was performed according to standard protocol (Harlow and Lane, 1988). About 500 mg of H and H stage 34 (Embryonic Day 8 or E8, Hamburger and Hamilton, 1951) chicken dorsal skin was used to prepare protein extracts. About 100 μg of protein extract was applied to each gel lane.

Gel Shift Assay

Two complementary 31mer oligonucleotides corresponding to positions −33 to −60 of the 5′ flanking region of rat somatostatin gene (Montminy *et al.*, 1986) were synthesized. The annealed oligonucleotide contained a cAMP response element (CRE), TGACGTCA, which in the context of the described flanking sequences have a high binding affinity for CREB (Deutsch *et al.*, 1988; Andrisani and Dixon, 1990). General methods for gel retardation assay and extraction of nuclear protein were used (Chodosh, 1987). About 500 mg of dorsal skins was dissected out from Day 8 embryos and protein was extracted according to Roy *et al.* (1991). The CRE fragment was end-labeled using [α-^{32}P]-dATP and Klenow fragment. About 30,000 cpm of the probe was mixed with 1 μg of protein extract in binding buffer. Bovine serum albumin (BSA) at a concentration of 5 mg/ml was included in the reaction mix to increase the CRE/CREB interaction (Zhang *et al.*, 1992). The mixture was then run on low ionic strength native polyacrylamide gel. The posi-

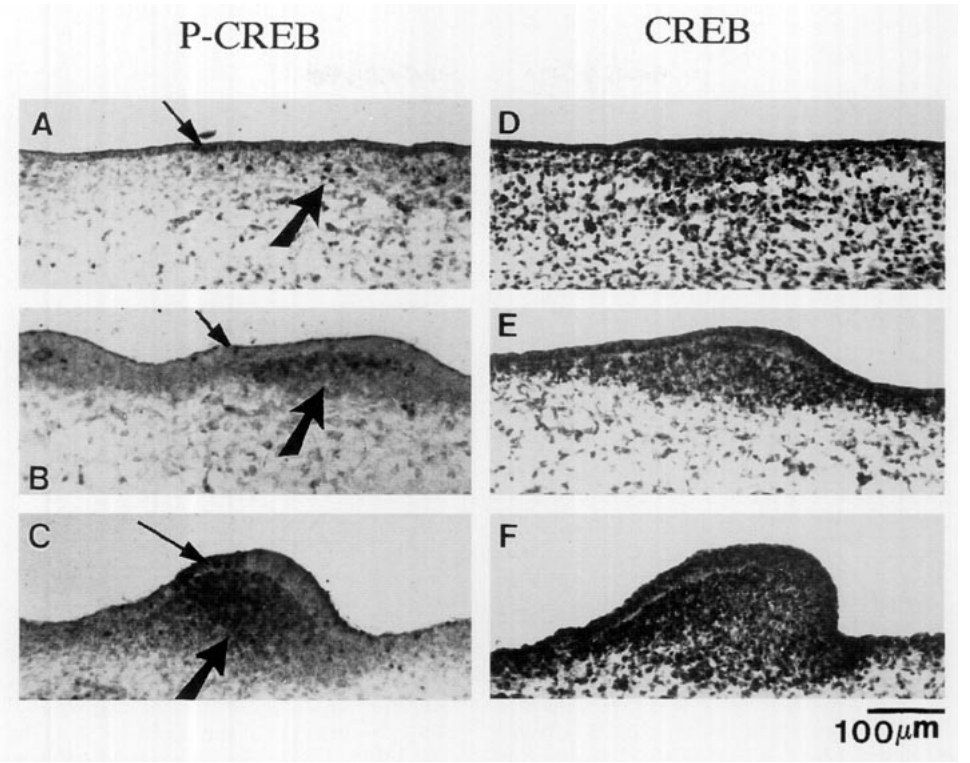


FIG. 3. Temporal and spatial expression of P-CREB and CREB during feather bud formation. Sagittal sections of dorsal skin. Anterior is to the left. Immunoalkaline phosphatase staining. (A–C) P-CREB. (D–F) CREB. (A, C,) Stage 29 (E6); (B, E,) stage 32 (E7.5); (C, F,) stage 34 (E8). CREB is ubiquitously expressed in both epidermis and dermis and in both bud and interbud areas in all of the stages examined. P-CREB is only expressed in the feather placode and feather bud region, in both epithelia and mesenchyme. Small arrows point to the expression in the epithelia. Note the round nucleus staining pattern. Big arrows indicate the enriched expression in feather mesenchyme. P-CREB staining represents the subset of CREB that gets phosphorylated. Size bar, 100 μm.

tion of the retarded band was visualized with autoradiography. When CREB or P-CREB antibodies were used, they were added to the protein extract at the same time that the probe was added.

Kinase Assays

PKC and PKA assays were performed according to manufacturer's protocols (Gibco BRL) on stage 31 (E7) and stage 34 (E8) chicken dorsal skin extracts, respectively. For the PKC assay an N-terminal acetylated synthetic peptide, derived from myelin basic protein, was used as specific substrate and a peptide from the regulatory domain of calcium-dependent PKCs (PKC (19–36)) served as a specific inhibitor of PKC. As recommended by the manufacturer, PKC was partially purified by passing the extracts through a DEAE–cellulose column (Whatman DE52) and eluting with a high salt solution. For the PKA assay the synthetic heptapeptide Kemptide, which has a PKA substrate consensus sequence, served as PKA-specific substrate and a synthetic 17-amino-acid peptide (PKI (17aa)), derived from protein kinase inhibitor, was used as a specific inhibitor of PKA. Incorporation of ^{32}P into substrates indicated the kinase activity of the extracts. The specimens were always run in duplicates and the difference was always less than 5%. Three independent experiments were carried out which showed a similar trend. We did not pool the data from these three independent experiments together because the absolute value varied. However, the relative relationship was similar. Therefore we showed one exemplary experiment which is a representative of six different determinations using extracts derived from three different extractions.

RESULTS

Distinct Expression Patterns of PKC, CREB, and P-CREB in Feather Development Shown by Immunolocalization

To explore intracellular signaling molecules involved in feather development, we began by examining the expression of several intracellular signaling molecules in stage 32 embryonic chicken dorsal skin (Embryonic Day 7.5). Among them, PKC, CREB, and P-CREB showed the most interest-

ing and distinct distribution patterns (Fig. 1). PKC is specifically localized in the interbud mesenchyme (Fig. 1A). In contrast, CREB is present in both epithelium and mesenchyme, in both feather germ and interfeather germ regions (Fig. 1B). P-CREB, representing the subset of CREB which is phosphorylated, is shown in a nuclear staining pattern with higher density in the feather germ region compared to that in the interfeather germ region (Fig. 1C). Interestingly, no overlap is seen between the sites of expression of PKC and P-CREB. The intriguing spatial distribution prompted us to look into the developmental course of this molecular expression.

We examined skin from the stage before feather placode can be discerned (stage 29) to the stage when a feather bud is well formed (stage 36). Surprisingly, in the developing skin we did not detect the presence of PKC before stage 30 (E6.5). The PKC antibody does stain other embryonic tissues in the same section (not shown). Around stage 30 (E6.5), PKC suddenly appears as a thin layer immediately beneath the dermal and epidermal junction and quickly expands downward to the whole layer of mesenchyme. This is followed by the periodic disappearance of PKC from mesenchyme immediately beneath the feather placode. The oval-shaped regions devoid of PKC gradually increase between stages 32 and 35 (E7.5–E9), and eventually PKC is limited to the interbud mesenchyme at stage 36 (E10). Throughout these stages (stages 30–36 or E6–E10), no significant expression of PKC is detected in epithelia within or outside the feather buds (Fig. 2).

CREB is ubiquitously distributed in both epithelium and mesenchyme, within and outside the feather germs during the examined stages, 30 to 36 (Figs. 3D, 3E, and 3F). In contrast, P-CREB, negative before stage 29 (E6), begins to be observed in the epithelia and in small clusters in mesenchyme (Fig. 3A). At stage 32 (E7.5), P-CREB is seen in placode epithelia. P-CREB is also expressed in the placode mesenchyme, but is completely absent in the interplacode mesenchyme (Fig. 3B). At stage 34 (E8), P-CREB is seen on the feather bud epithelia and is enriched in the feather bud mesenchyme, but is absent in the interbud epithelia and mesenchyme (Fig. 3C). The appearance of P-CREB in mesenchyme is transient, and by stage 36 (E10) P-CREB disappears in the mesenchyme but remains in the feather bud epithelium (not shown). To further characterize these signaling molecules in developing skin, we did immunoblots and determined kinase activities.

Immunoblots of PKC and P-CREB and Activities of PKC and PKA

We used Western blots to detect the expression of PKC isoforms in chicken dorsal skin during stage 34 (E8) of embryonic growth. Chicken PKC has not been previously characterized. Results using the UBI PKC antibody showed the presence of a single band around 116 kDa (Fig. 4A). The relative molecular mass of chicken PKC is higher than that of the mammalian PKC (77 kDa) (Stabel and Parker, 1991)

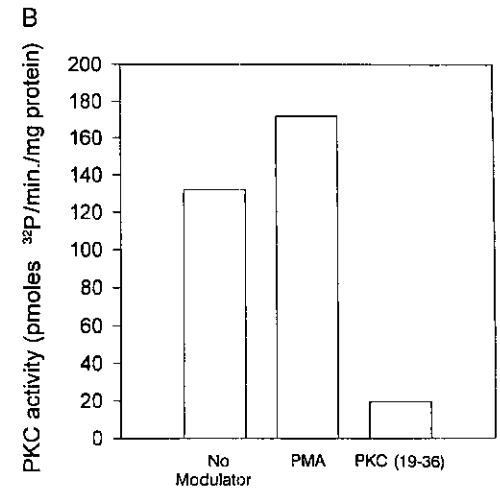


FIG. 4. PKC immunoblot and activity in developing skin. (A) Immunoblot of PKC using stage 34 (E8) dorsal skin extract. A single band at around 116 kDa can be observed. (B) PKC activity of stage 34 (E8) dorsal skin extracts. To measure PKC activity, PKC-specific substrate (Gibco BRL) was incubated with skin extracts in the absence of any modulators (no modulator), in the presence of PKC activator (PMA) and in the presence of PKC inhibitor (PKC (19–36)). This graph is a representative of six different determinations using PKC derived from three independently prepared extractions.

and Xenopus PKC (85 kDa) (Otte and Moon, 1992). However, 115-kDa species have also been observed (Saxon *et al.*, 1994), and PKCu has recently been found to be 115 kDa (Johannes *et al.*, 1994). In rabbit all of the classical PKC isozymes (α, β, γ) have the same mobility (Hagiwara *et al.*, 1990); thus it is also possible that the observed single band may contain more than one isozyme.

To determine whether physiological PKC activity exists in embryonic skin, PKC assays were done on stage 34 (E8) chicken dorsal skin extract in the absence and the presence of PKC activator or inhibitor (Fig. 4B). This showed that the stage 34 (E8) extract has a substantial PKC activity (the bar graph designated as no modulator). Using the PKC-specific inhibitor (PKC (19–36), Gibco BRL), it was shown that 15% of this activity is PKC-nonspecific. Taking the PKC-nonspecific activity as background, PKC-specific activity could be calculated to be around 113 pmoles phosphate transferred/min/mg total protein. In the presence of PKC-specific activator PMA (phorbol 12-myristate 13-acetate), PKC-specific activity was increased by 35%, suggesting that at this stage, 35% of PKC isozymes are inactive. It has to be pointed out that the activity shown here represents the average of the whole skin extract. Thus, at this time the immunocytochemical observation still represents a finer resolution regarding the expression of PKC during feather morphogenesis. To have an *in situ* map of PKC activity, we will await the development of a PKC histochemical assay in the future.

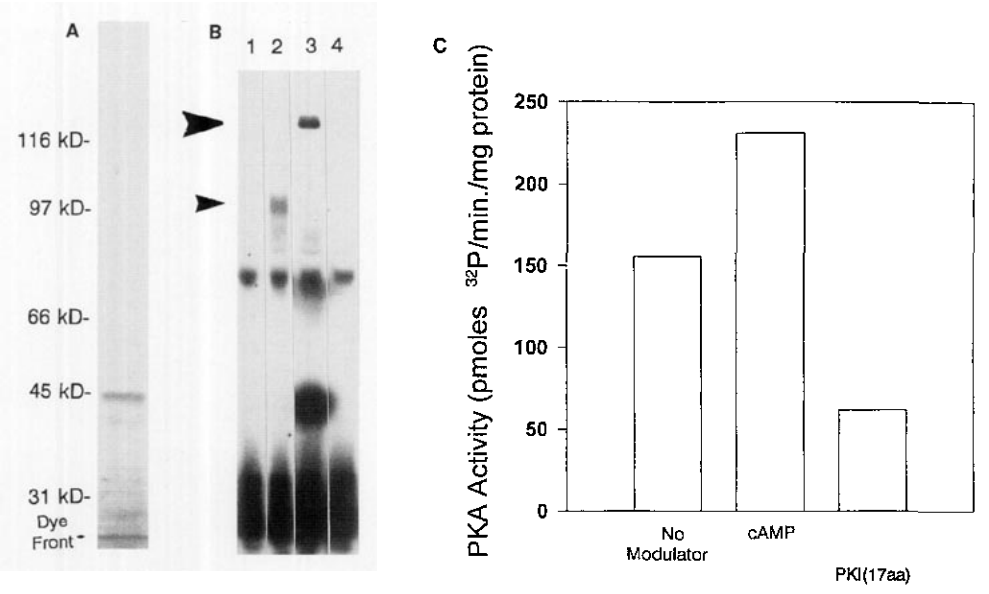


FIG. 5. P-CREB immunoblot, gel shift, and PKA activity in developing skin. (A) Immunoblot of P-CREB using stage 34 (E8) dorsal skin extract. A prominent band at around 45 kDa can be observed. (B) Gel shift assay. All lanes contain ^{32}P end-labeled somatostatin CRE probe. (Lane 1) Probe only. (Lane 2) Stage 34 (E8) extract. A retarded band composed of probe–CREB complex can be observed (small arrowhead). (Lane 3) Stage 34 (E8) extract incubated in the presence of 0.2 μg anti-CREB antibody. The disappearance of probe–CREB complex and appearance of a new supershifted band composed of probe–CREB–anti-CREB can be observed (large arrowhead). (Lane 4) Stage 34 (E8) extract incubated in the presence of 0.2 μg antibody to P-CREB. A shifted band cannot be observed, indicating interaction of the antibody with P-CREB. In addition, no supershifted band is observed. (C) PKA activity of stage 31 (E7) dorsal skin extract. To measure PKA activity, PKA-specific substrate (Gibco BRL) was incubated with skin extracts in the absence of any modulators (no modulator), in the presence of PKA activator (cAMP) and in the presence of PKA inhibitor (PKI (517aa)). This graph is a representative of six different determinations using extracts derived from three different extractions.

Immunoblot of P-CREB in stage 34 (E8) embryonic chicken dorsal skin showed the presence of a prominent band of about 43 kDa (Fig. 5A). To determine whether there is CRE binding activity in developing skin, we did a gel shift assay using a somatostatin CRE DNA fragment and stage 34 (E8) extracts (Fig. 5B, lane 2). Only one shifted band could be observed with stage 34 (E8) extract. A cold somatostatin CRE DNA fragment or a CRE fragment from a human choriogonadotropin α promoter could both eliminate the shifted band. However, neither a somatostatin DNA fragment that had a mutant CRE (TGAAGCCA instead of TGACGTCA) nor a TRE fragment from a collagenase promoter could compete with the wild-type somatostatin fragment to eliminate the shifted band (not shown). We tested anti-CREB and anti-P-CREB antibodies to see if they can supershift the shifted band. Upon addition of anti-CREB antibodies a supershifted band could be observed, demonstrating the presence of an anti-CREB/CREB/CRE complex, while the CREB/CRE gel shift disappeared (Fig. 5B, lane 3). No supershift was seen when anti-P-CREB antibody was used because the anti-P-CREB antibody interfered with the formation of the P-CREB/CRE complex (Fig. 5B, lane 4). The reaction of antibodies with the shifted band proved that the molecular species we detected with immunocytochemical methods were indeed CREB and P-CREB. The gel shift result along with that of the immunoblot suggests that there is only one CREB species in developing skin.

To see whether physiological PKA activity exists in developing skin, we determined the PKA activity of E7 (stage 31) skin extract in the absence and the presence of PKA activator or inhibitor (Fig. 5C). Assuming that the PKA activity of the extract in the presence of the PKA inhibitor (PKI (17aa)) corresponds to background, PKA-specific activity was around 94 pmoles ^{32}P transferred/min/mg total protein. Upon addition of PKA-specific activator (cAMP), the PKA-specific activity was increased by about 80%, suggesting that 80% of the PKA isozyme(s) is physiologically present in an inactive state.

The above results showed that P-CREB and PKC are expressed in feather buds and that there are physiological PKA and PKC activities in developing skin. To test their functional roles, we used pharmacological agonists and antagonists and a chicken skin explant culture model (Figs. 6A and 6B) for analyses.

PKA Agonists Enhance the Formation of Dermal Condensations

Forskolin, an activator of adenylyl cyclase, was added to stage 34 (E8) skin culture medium at a concentration of 20

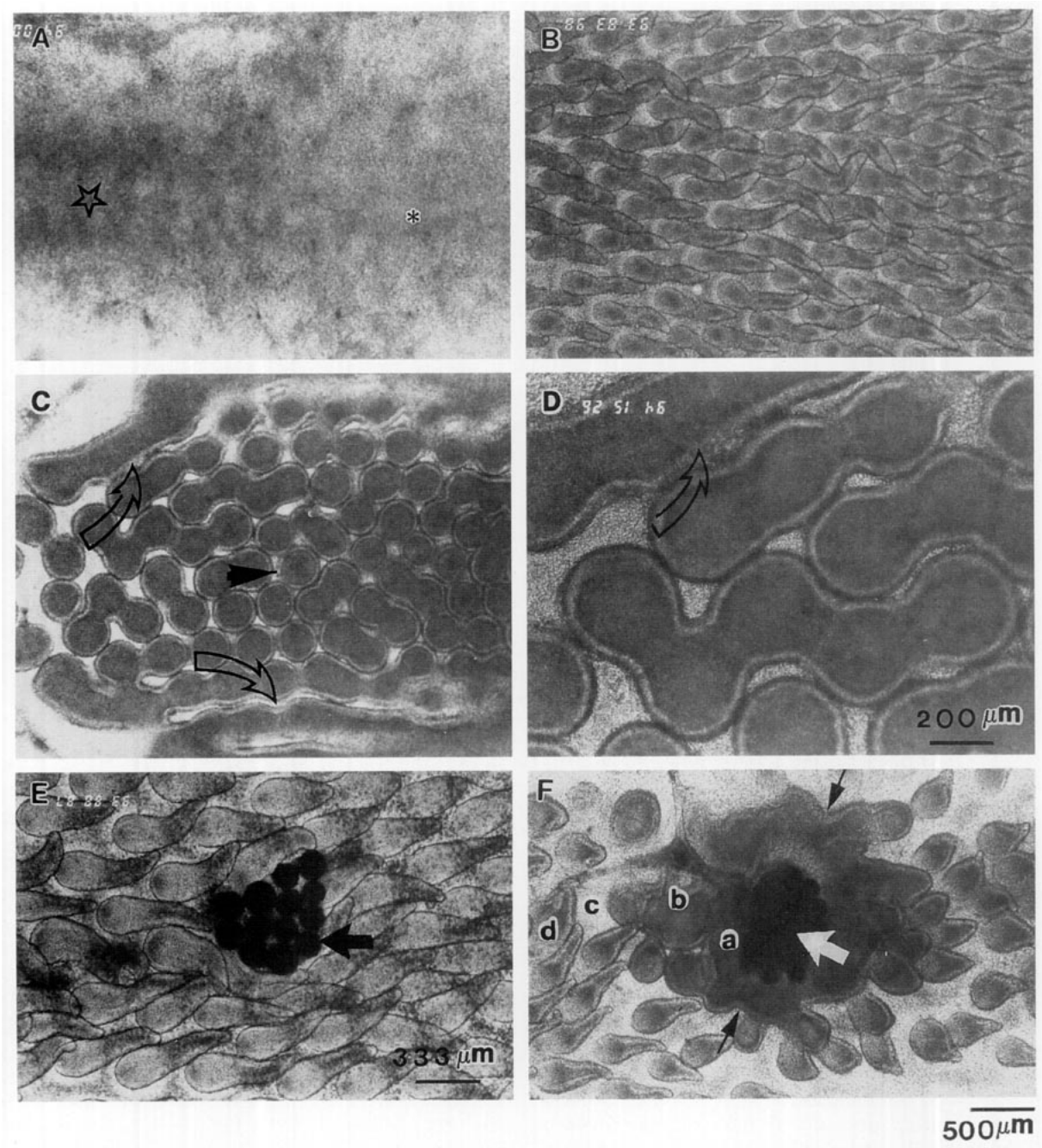


FIG. 6. The effect of PKA activators forskolin and 8(4CPT)cAMP on feather bud condensation. Whole-mount view of the skin explant. Anterior is to the left. (A) Normal stage 30 (E6.5) chicken dorsal skin. Cells in posterior (right) region are still homogeneously distributed (region marked by asterisk). Initial formation of dermal condensations can be observed in the anterior (left) midline region (marked by star). (B) Normal stage 34 (E8) dorsal skin showing feather bud growth after 5 days of incubation in DMEM at 37°C. (C) Forskolin-treated stage 34 (E8) skin after 5 days of incubation in DMEM at 37°C. Forskolin (20 μM) causes the formation of round, big, and nonelongating feather buds (arrowhead). Feather buds farthest from the central longitudinal axis are youngest and are affected more dramatically, forming a continuous condensed zone (open curved arrow). (D) Similar to C, high-power view to show the fused round buds. (E) Stage 31 (E7) skin explant after 5 days of incubation in DMEM at 37°C. Multiple beads (straight thick arrow) presoaked in DMEM (the solvent for 8(4CPT)cAMP) have no effect on feather development. (F) Same as E except 8(4CTP)cAMP presoaked beads were used. 8(4CPT)cAMP presoaked beads (straight thick arrow) produced a perturbed phenotype with four zones surrounding the beads. Immediately next to the beads are fused dermal condensations (a). Next to it is a rim of enlarged buds (b and thin arrow). Outside this is a zone composed of less dense mesenchymal cells (c). Outside this are normal buds (d). Size bar, A–C and F, 500 μm; D, 200 μm; E, 333 μm.

TABLE 2
The Effect of PKA and PKC Signal Transduction Pathway Modulators on Feather Bud Placode Size in Stage 34 (E8) Skin Explants

Enzyme	*Drug*	*Concentration*	*No. of Trials*	*Size of buds*[b]	*Consistency*
—	dbcGMP	1 m*M*	12	Normal	100%
Prot. Kin. A	dbcAMP (A)[a]	1 m*M*	20	+++	95%
Prot. Kin. A	8(4CPT) cAMP (A)	50 μM	4	+++	100%
Aden. Cycl.	Forskolin (A)	1 μM	4	Normal	100%
		5 μM	4	+	100%
		10 μM	10	+++	100%
		20 μM	96	++++	90%
		50 μM	4	+++	75%
Prot. Kin. A	H8 (I)	1 μM	4	Normal	100%
		10 μM	4	Normal	100%
		20 μM	80	+ and –[c]	85%
		50 μM	12	No buds	100%
P. diesterase	Ro20-1724 (I)	0.2 m*M*	6	+	66%
Prot. Kin. C	Calphostin C (I)	20 n*M*	4	Normal	100%
		200 n*M*	20	+	70%
Prot. Kin. C	B-PDiAc. (A)	100 n*M*	40	– – –	85%
		1 μM	20	No buds	90%
Prot. Kin. C	PMA (A)	100 n*M*	20	– – –	80%
		1 μM	20	No buds	80%

[a] I stands for inhibitor and A stands for activator of each enzyme.
[b] Semi-quantitation of the size of dermal condensations: (+), Each plus sign indicates a 25% increase in diameter of placode; (–), Each minus sign indicates a 25% decrease in diameter of placode.
[c] At 20 μM concentration there are some buds which appear larger and some which appear smaller than normal.

μM and skin explants were cultured for 5 days. Round and dense buds were observed instead of elongated feather buds (compare Figs. 6B and 6C). Dibutyryl cAMP (db-cAMP) and 8(4CPT)-cAMP had similar effects. Control skin explants and cGMP-treated explants appeared normal (Table 2).

The response of skin explant to forskolin is stage dependent. When skin explants from stages 30–31 (E6.5–7) were incubated with forskolin, they did not form distinct dermal condensations but formed a confluent dermal cell condensation similar to that of Fig. 9B. When explants from stages 35–36 (E9–E10) were incubated with forskolin, the effect became less obvious. Because feather buds initiate from the midline and spread to the lateral, a stage 34 (E8) skin exhibits different stages of feather development. The younger buds at the lateral sides exhibited the effect of forskolin on early skin, showing the fused or confluent dermal condensations (Figs. 6C and 6D). We have also exposed stage 34 (E8) skin to cAMP (1 m*M*) or forskolin (20 μM) during only the first day of culture. After 5 days the results were very similar to those explants cultured in the presence of forskolin for 5 days. Addition of forskolin (20 μM) to stage 34 (E8) explants when they had been already cultured with no drugs for 2 days had only a minor effect on the normal phenotype (not shown). Together, the above results show that younger skins are more susceptible to the effect of forskolin.

To analyze the effect of a local gradient, we soaked anion exchange beads in cAMP analog 8(4CPT)-cAMP (Miller *et al.*, 1975) and placed them on top of stage 31 (E7) skin explants. Alteration of dermal cell distribution can be seen in four different zones around the beads (Fig. 6F, a–d). Immediately surrounding the beads are fused dermal condensations, followed by enlarged buds, then a halo zone, and finally normal buds (Figs. 6E and 6F). The data suggest that mesenchymal cells are attracted toward this cAMP analog to form enlarged condensations and buds near the beads. The redistribution of mesenchymal cells led to the observed halo zone with fewer cells. Multiple beads are required to deliver enough 8(4CPT)-cAMP for this effect.

We then tested the effect of the PKA inhibitor N-(2-(methylamino)ethyl)-5-isoquinolinesulonamide dihydrochloride (H8). Twenty micromolar H8 was added to the skin culture media (Table 2). As with forskolin, the effect is more obvious in younger skin explants. Application of H8 to stage 31 (E7) skin caused inhibition of feather buds in the lateral region of the skin explant and big, small, or missing buds in the central regions of the explant (Fig. 7A). Application to the stage 34 (E8) skin had a similar but less severe effect (Fig. 7B). Application of 50 μM H8 to stage 32 (E7.5) skin resulted in a skin that appeared flat (Fig. 7C, peripheral region marked with star). Time course observation showed that dermal condensation is a reversible process. At higher doses, H8 can make performed dermal condensations disappear (Figs. 7D–7F).

Since H8 may also inhibit PKC, we further tested whether H8 activity is through inhibition of PKC or PKA. When PKA activator 8(4CPT)-cAMP-soaked beads were placed on

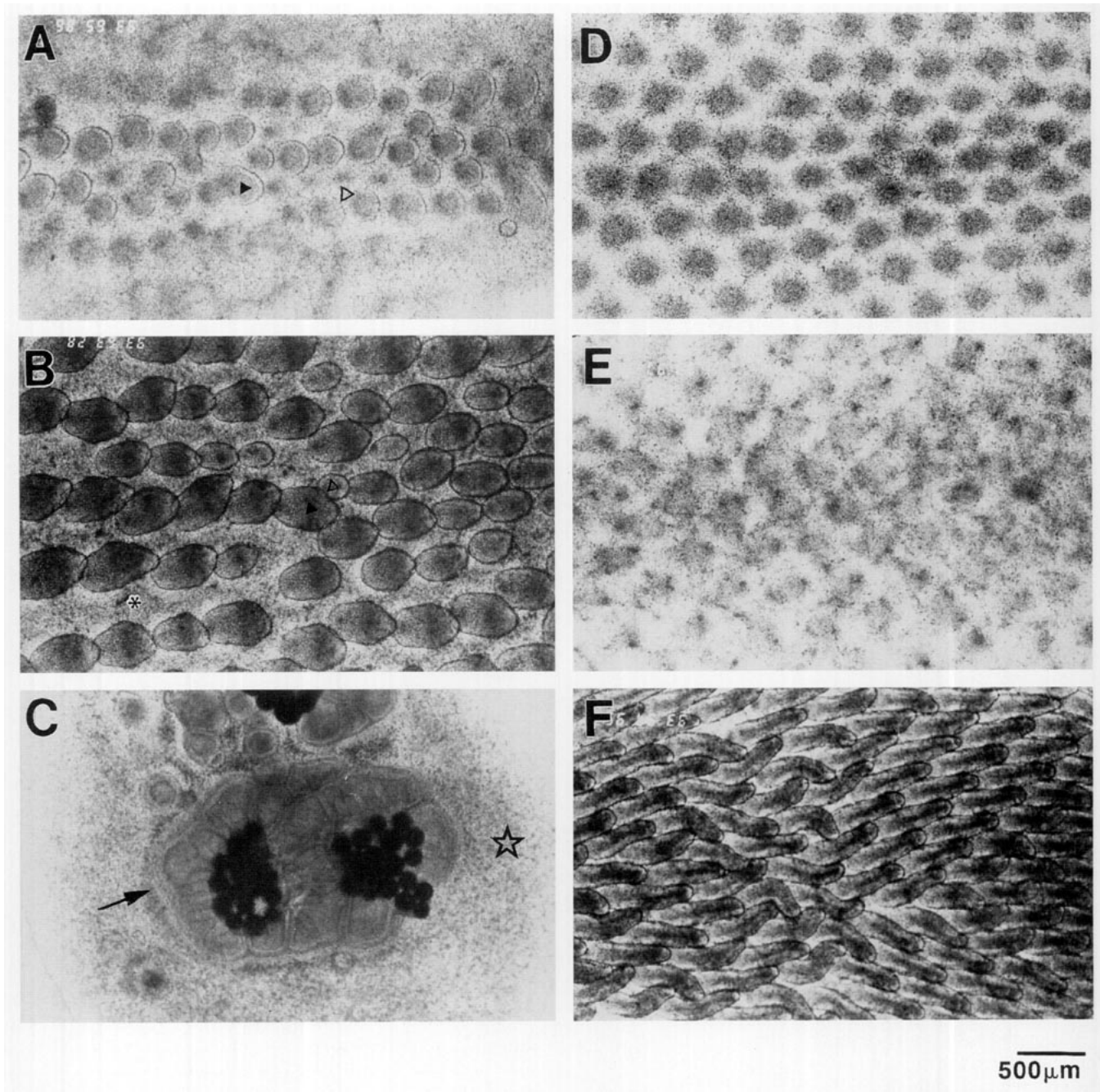


FIG. 7. The effect of PKA inhibitor H8 on feather bud formation. Whole-mount view of the skin explant. Anterior is to the left. (A) Stage 31 (E7) skin explant after 2 days in the presence of 20 μM H8. The growth of young buds in both lateral zones (upper and lower part of the explant) were inhibited. The buds in the central rows are big (solid triangle), small (open triangle) or missing. (B) Stage 34 (E8) skin explant after 5 days of growth in the presence of 20 μM H8. Note the uneven sizes and missing rows (*) of buds (compare with normal Stage 34 (E8) buds, Fig. 6B). (C) 8(4CPT)cAMP-soaked beads were placed on stage 32 (E7.5) skin explants which were treated at the same time with 50 μM H8 (dissolved in media). 50 μM H8 completely inhibits the formation of buds (star), but groups of fused buds were induced around the 8(4CPT)cAMP-soaked beads (arrow). (D–F) Disappearance of dermal condensation following H8 treatment. (D), Stage 34 (E8) skin explant, 50 μM H8, Day 0. (E) The same culture as in D after 4 days of culture showed that dermal condensations disappear. (F) Control stage 34 (E8) explant culture for 4 days showed the well-grown feather buds. Size bar, 500 μm.

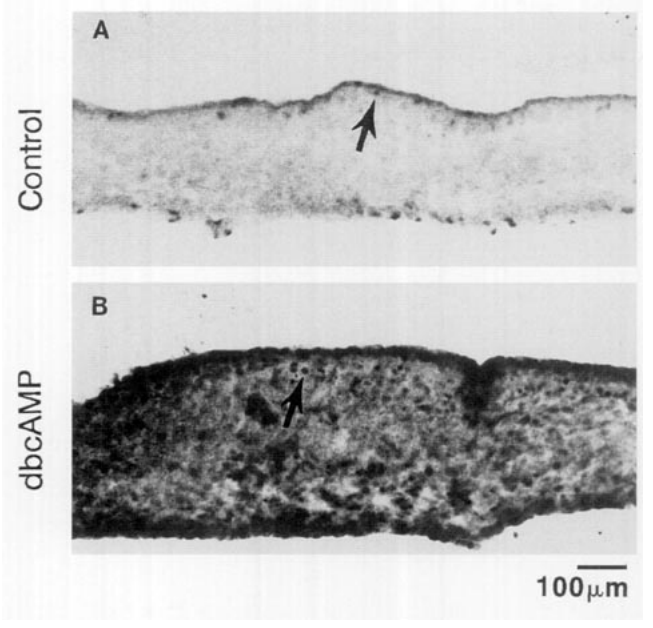


FIG. 8. The effect of dbcAMP on CREB. Sagittal sections of dorsal skin explant. Anterior is to the right. Stage 31 (E7) skin explant was cultured without (A) or with (B) dbcAMP (1 m*M*) for 4 hr, then fixed, and sectioned. Note that at this stage there are only a few P-CREB-positive nuclei in the epithelium. The mesenchyme is mostly negative. A large amount of P-CREB staining appeared in both epithelium and mesenchyme after dbcAMP stimulation. Arrow points to the nucleus staining pattern of P-CREB. Bar, 100 μm.

top of a stage 32 (E7.5) skin treated with 50 μ*M* H8 (Fig. 7C), the effect of H8 was reversed and fused condensations formed around the beads (also compare with Fig. 6F). This result suggested that H8 exerts its effect mainly through inhibition of PKA.

To test whether dbcAMP induces the expression of P-CREB in these skin explant cultures, we added 1 m*M* dbcAMP to stage 31 (E7) skin explant media for 4 hr and harvested the skin explants. The explants were fixed and sectioned. We observed a large increase of P-CREB in the explants (Fig. 8), which is consistent with our other data.

Although PKA agonists enhanced the formation of dermal condensations, the forskolin-stimulated buds became round and stunted and could not elongate (Figs. 6C, 11E, and 11F). Feather development is a multistep process. PKA activity is needed in the dermal condensation stage. In the feather bud elongation stage, PKA probably has to be suppressed to allow the growth of feather buds.

PKC Agonists Enhance the Expansion of the Interbud Domain

The effect of PKC on dermal condensations was analyzed by culturing in the presence of PKC agonists and antagonists (Table 2). In stage 34 (E8) skin explants, 4 β-phorbol-12,13-diacetate (β-PDiac.) caused the formation of very small buds at low concentrations (100 n*M*) (Fig. 9A) and complete inhibition at high concentrations (1 μ*M*). PMA had an identical effect. The skin explant appeared thinner because some cells migrated out of the explant. However, the remaining buds and explant continued to grow even after 6 days in culture. Calphostin C, a specific inhibitor of PKC, had no obvious effect when added to the explant by itself at 20 n*M*, but slightly promoted feather bud growth at 250 n*M* (not shown). When 100 n*M* calphostin C along with a low concentration of forskolin (5 μ*M*) was added to stage 34 skin explants, it remarkably promoted the fusion effect of dermal condensations (compare Figs. 9B and 6C). There is extensive fusion in the midline region and in the lateral region. Five micromolar forskolin had only a small effect on dermal condensation (Table 2). This suggests that calphostin acts synergistically with forskolin to enhance the formation of dermal condensations.

The results of the many experiments and dose effect of the pharmacological reagents are listed in Table 2. Overall, enhancement of PKA or suppression of PKC led to enhanced feather bud regions, while suppression of PKA or enhancement of PKC led to enhanced interbud regions.

N-CAM Is One of the Downstream Targets of PKA and PKC

What downstream molecules might be altered by the PKA and PKC activity which then lead to alteration in dermal condensations? It is reasonable to search for adhesion molecules that have been shown to be involved in feather morphogenesis. We have found previously that N-CAM can mediate the formation of dermal condensations (Jiang and Chuong, 1992) and is a reasonable candidate to study. We applied both dbcAMP (1 m*M*) and phorbol ester, PMA (100 n*M*), to stage 32 (E7.5) skin explant cultures and examined expression of N-CAM using whole-mount antibody staining. At this stage the expression of N-CAM can be transiently seen in feather bud mesoderm and the epithelia in the tip of the growing feather bud (Fig. 10A). Following cAMP treatment, N-CAM staining is diffusely distributed among mesenchymal cells that form fused branching dermal condensations (Fig. 10B). There are also regions in which N-CAM expression appears as a whole sheet. Sections of the cAMP-treated skin showed stunted feather buds with thickened epithelia (Figs. 11A, 11B, 11E, and 11F). The distribution of N-CAM became expanded and diffuse in the dermal regions (Figs. 11C and 11D). In contrast, following treatment with PMA, N-CAM disappeared from lateral skin that was at the precondensation stage at the time of treatment. These N-CAM-negative areas did not form feather buds. Feather buds along the midline were older but now became aborted small feather buds with intense N-CAM staining (Fig. 10C).

DISCUSSION

Our aim in this study is to explore the molecular mechanism in secondary induction using feather morphogenesis

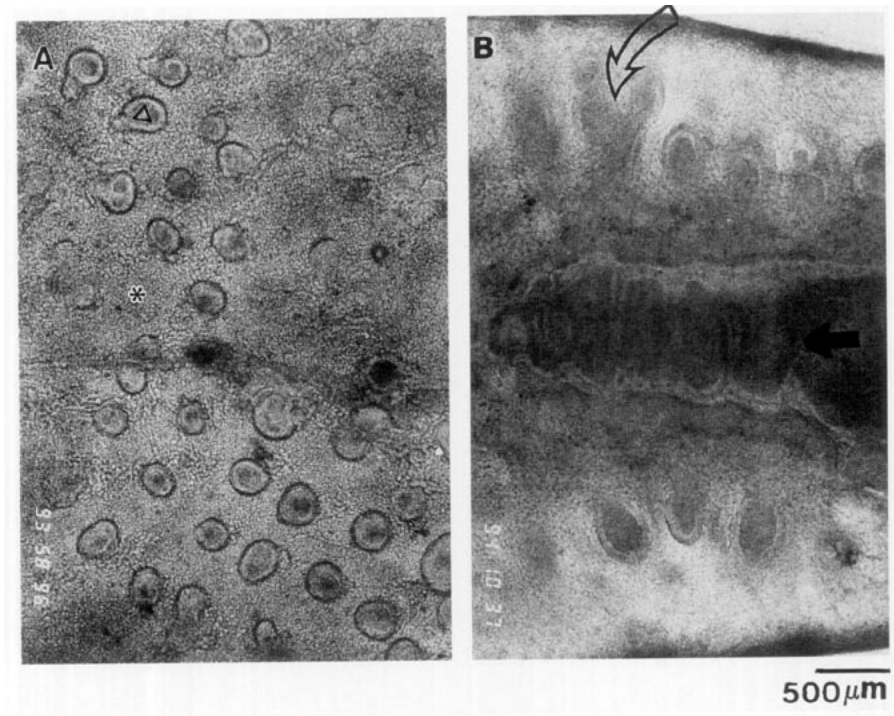


FIG. 9. The effect of PKC modulator on feather bud formation. Whole-mount view of the skin explants. (A) Stage 34 (E8) skin explant after 5 days in the presence of PKC activator PMA (100 n*M*). Note the small feather buds (triangle) and the expanded interbud region (*). PMA suppresses dermal condensation. (B) PKC inhibitor calphostin (100 n*M*) augments the dermal condensing effect of forskolin (5 μM). A broad fused zone of condensed dermal cells forms along the midline (straight arrow). Several ongoing fusions can be seen in the lateral zone (curved arrow). Size bar, 500 μm.

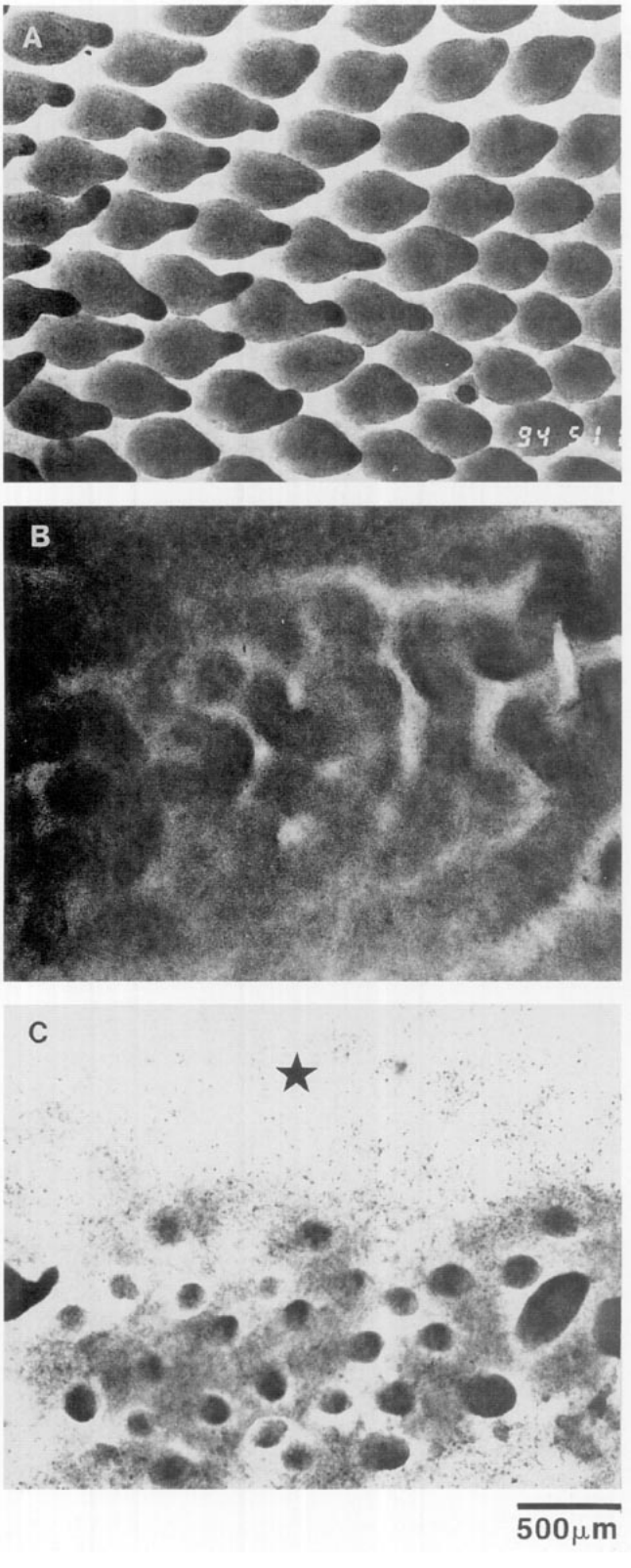
as a model. Although detailed events in secondary inductions may vary, unifying principles can be learned from studying skin appendage inductions and other secondary inductions (Riddle *et al.*, 1993) and comparison with primary inductions (Melton, 1991). Skin appendages are remarkable in their regular periodicity, distinct morphology, and accessibility to experimental manipulation (Sengel, 1976). Although many molecules including growth factors, adhesion molecules, matrix molecules, homeobox genes, and oncogenes are expressed or involved in feather morphogenesis (reviewed in Chuong, 1993), signal transduction following tissue interactions in skin development has been under-studied. In this study we focused on the alterations of intracellular signaling which occur in the early phase of feather induction and identified some alterations associated with the formation of dermal condensations.

Concerted Reciprocal Waves of PKA and PKC Activities and the Formation of Alternating Dermal Condensation and InterBud Regions

The expression patterns of PKC, CREB, and P-CREB in developing skin as detected by immunocytochemical methods (Figs. 1–3) show that both PKC and P-CREB were initially absent. PKC suddenly increases when feather germs start to form. In regions of feather germ formation, PKC decreases, while P-CREB increases. Subsequently, PKC is expressed in the interbud region, while P-CREB is expressed in the bud epithelium. CREB is ubiquitous during all of these times. The specificity of the antibodies is shown by immunoblot and gel supershift (Figs. 4 and 5). The presence of PKA and PKC in the developing skin extracts was further corroborated by kinase assays that showed the presence of PKA and PKC activity.

These results prompted us to raise the hypothesis that enhancement of PKA activity favors dermal condensations while enhancement of PKC activity suppresses the formation of dermal condensations and favors the formation of interbud dermis. Experiments with PKA and PKC modulators in skin explant cultures are consistent with this notion. PKA activators and PKC inhibitors expand regions occupied by dermal condensations, while PKA inhibitors and PKC activators expand regions occupied by interbud dermis (Figs. 6, 7, and 9).

It has been shown that cAMP acts through PKA to phosphorylate CREB (Montminy *et al.*, 1990; Lee, 1991; Karin, 1992) and enhance its transcriptional activity (Yamamoto *et al.*, 1988; Gonzalez and Montminy, 1989), although CREB



can also be activated by other kinases (Lee *et al.*, 1990; Gonzalez *et al.*, 1989; Dash *et al.*, 1991; Sheng *et al.*, 1991). Our immunostaining data also showed that after incubation of dorsal skin explants in the presence of dbcAMP, the level of phosphorylated CREB increases dramatically (Fig. 8). Since CREB protein is ubiquitously present in feather buds, the level of P-CREB may rely on the equilibrium of kinase and phosphatase activities that act on CREB. Thus, the regulation of the CREB pathway appears to be at the posttranslational level.

PKC is activated when translocated from cytoplasm to cell membrane (Olson *et al.*, 1993). However, in this study we determined the total PKC activity of stage 34 (E8) dorsal skin extract; thus the measured activity is that of the homogenate of whole skin. Unlike studies on cell lines, biochemical data like this are hard to interpret in terms of histogenesis, in which the activity present in bud or interbud regions is an issue. We will pursue the determination of *in situ* activity of PKC in the future when specific PKC histochemistry is available.

Most drugs used in this study were specific activators or inhibitors of each pathway. However, some may not be absolutely specific. For example, H8 can also inhibit PKC, but is required at a much higher concentration (Hidaka *et al.*, 1984). We chose a concentration that should maximally inhibit PKA but have a minimal effect on PKC. This was further verified by using cAMP analog, 8(4CPT)-cAMP, which reversed the observed effect of H8 in these experiments (Fig. 7C).

Potential Regulators and Targets of Protein Kinases during Skin Appendage Development

What then can turn up PKA or turn down PKC in developing feather germ? The formation of skin appendages is the result of epithelial–mesenchymal interactions and the two components are indispensable to each other. When the epithelium is stripped away, the remaining mesenchyme fails to form dermal condensations, and if any dermal condensation has already formed, it will disappear. In these epithelium-free dermis, P-CREB staining disappears and PKC expression becomes ubiquitous (Ting-Berreth and

FIG. 10. The effect of PKA and PKC activation on N-CAM expression. Whole-mount immunostaining of NCAM. Whole-mount view of the stained skin explants. Stage 34 (E8) skin was cultured for 4 days. (A) Control. (B) Forskolin, 20 μM. (C), PMA, 100 nM. In the presence of forskolin, NCAM becomes diffusely distributed. Along the body midline (from 9 o'clock to 3 o'clock), the fused dermal condensations are all positive of NCAM. The fusion is more pronounced in the lateral regions, where cells are all NCAM-positive. In the presence of PMA, in the lateral regions (marked by a star), NCAM is completely suppressed and feather buds are also inhibited. Along the midline where feather buds are already formed, feather buds become shrunk small cellular aggregates which are highly NCAM-positive. Bar, 500 μm.

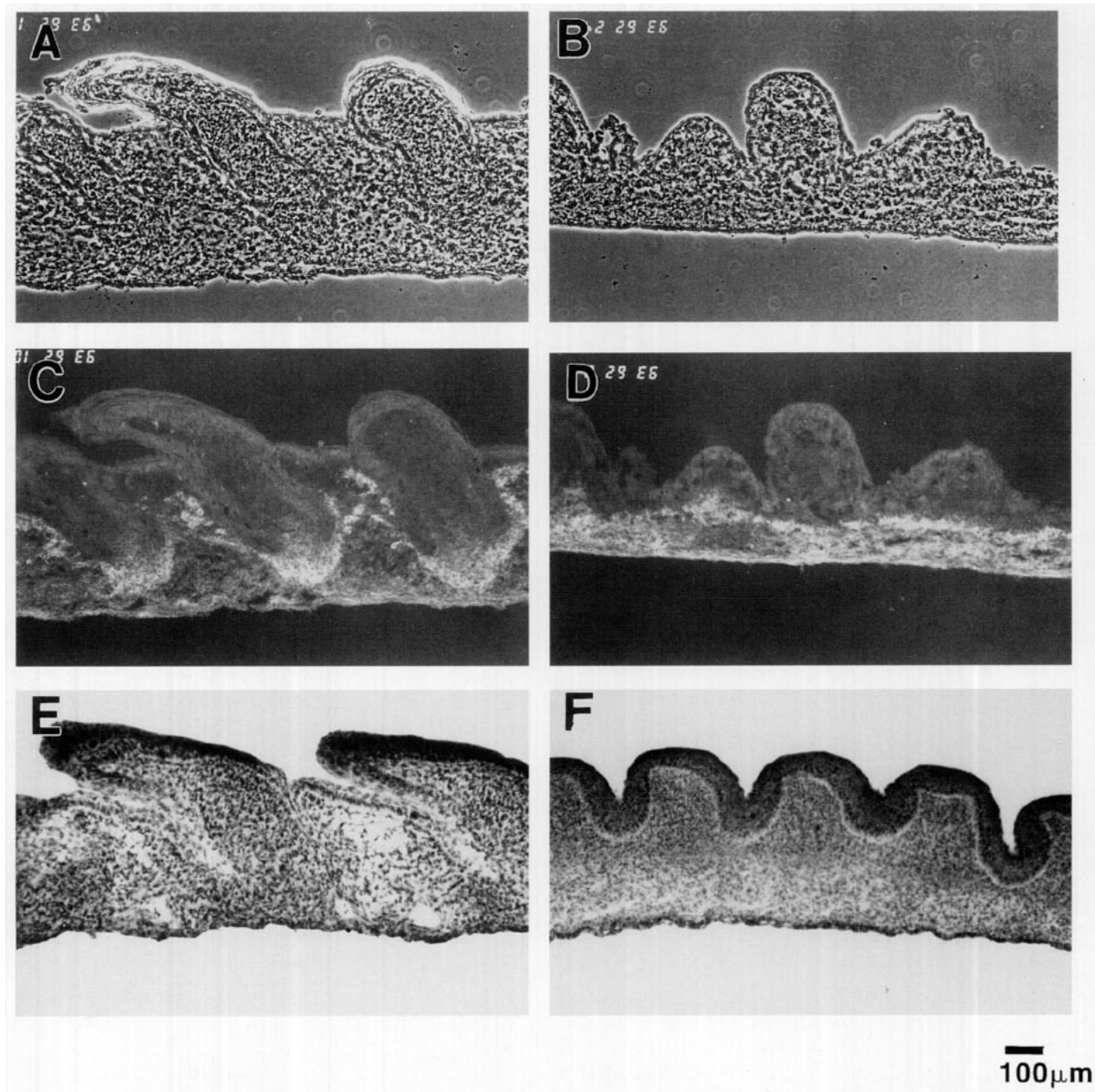


FIG. 11. Activation of PKA in developing skin caused disturbance of N-CAM expression. Sagittal section of cultured skin explant. Anterior is to the right. Stage 34 (E8) chicken dorsal skin with 10 μM forskolin for 3 days. (A, C, E) Control. (B, D, F) Skin explant treated with 10 μM forskolin. (A, B) Phase contrast; (C, D) immunofluorescent staining with anti-N-CAM; (E, F) H & E staining. Note that in the forskolin-treated feather explants, N-CAM becomes diffusely distributed in the dermal region. Also note the increased thickness of epithelia in forskolin-treated skin. Size bar, 100 μm.

Chuong, submitted for publication), suggesting that factors from placode epithelia are required to regulate PKA and PKC activities in mesenchyme beneath the placode. PKA is activated by adenylyl cyclase that is usually activated by the seven transmembrane receptor family members through G proteins (Gilman, 1984; Dohlman *et al.*, 1991). PKC is activated by phospholipase C-β, which in turn is activated by many membrane receptors (Huang *et al.*, 1989). In the future, we will search for the physiological activators of PKA and suppressors of PKC in developing skin.

It is also important to learn about the downstream events following the activity of PKA and PKC. P-CREB can regulate

the transcription of genes that have a CRE in their regulatory region (reviewed in Montminy *et al.*, 1990; Lee, 1991). In growing feather germs P-CREB may induce altered expression of a series of specific genes in mesenchymal cells whose expression eventually leads to the formation of condensed mesenchyme. Activation of PKC may lead to alteration of intracellular signaling, which influences cell motility and adhesion and leads to the dissolution of condensed mesenchyme. Among the likely candidates are adhesion molecules. We have earlier shown that N-CAM mediates dermal condensation (Jiang and Chuong, 1992). Activation of PKA causes diffused N-CAM expression. Phenotypically, there is fusion of dermal condensations into dermal stripes (Fig. 10). In contrast, suppression of PKA leads to enlarged, small, or missing buds, a phenotype very similar to the effect of anti-N-CAM Fab (compare Fig. 7B with Jiang and Chuong, 1992, Fig. 8G). Activation of PKC leads to suppression of N-CAM and also to feather germ formation. Thus modulation of PKA and PKC activity may regulate, directly or indirectly, downstream adhesion molecules such as N-CAM and others to mold the position and size of dermal condensations.

Another aspect of the effect of forskolin is that it prevents feather buds from elongating (Figs. 6C, 11E, and 11F). We have recently shown that homeobox genes *Msx-1* and *Msx-2* are expressed in the growing feather buds and that forskolin can suppress the expression of *Msx-1* and *Msx-2* (Noveen *et al.*, 1995). PKA activity may be required in the dermal condensation stage. When the feather buds progress into the next stage, PKA activity has to be tuned down to allow the elongation of feather buds. This is consistent with our observation that *in vivo* P-CREB expression in feather bud mesenchyme is transient. Enhanced PKA activity would "freeze" the buds at the dermal condensation stage.

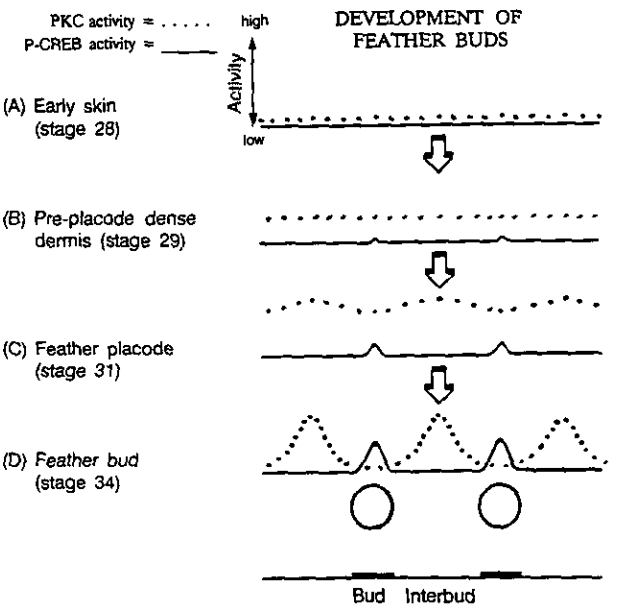


FIG. 12. Hypothetical model of progressively later stages of PKC and P-CREB activity during feather bud development. The height of the lines represent the level of activity of either PKC or P-CREB. The activities are superimposed along the longitudinal axis of skin (a line cut across three interbud and two feather bud domains). (A) In the early skin, both the PKC and P-CREB activity are low. (B) In the preplacode dense dermis, the PKC activity uniformly increases along the length of skin. The P-CREB activity appears at the site of the future placode. (C) At the feather placode stage, PKC activity continues to increase in the interbud domains but decreases in the placode domains. P-CREB activity increases only in the placode domains. (D) At the feather bud stage, PKC activity occurs mainly in the interbud domains while P-CREB activity occurs mainly in the bud domains. Corresponding sites of feather germ are shown as circles.

Model for the Formation of Feather Germs

With the presence of reciprocal PKA and PKC waves, is it the appearance of PKA or the disappearance of PKC that is pivotal to the formation of feather germs? Our data suggest that both pathways work in parallel. The PKA pathway may be a stronger signal for the formation of dermal condensations, while suppression of PKC can also lead to the formation of feather germs. Both agonistic and antagonistic activity between PKA and PKC have been observed in cultures under different conditions (agonistic: Otte *et al.*, 1989; Sassone-Corsi *et al.*, 1990; antagonistic: Shea *et al.*, 1992). Whether cross-talk exists between the two pathways in developing skin remains to be seen. For skin appendage induction, we present a working model that reveals part of the molecular chain of events in this induction pathway. We propose that the initiation, propagation, and termination of dermal condensation are regulated by the relative activity of PKA and PKC in mesenchymal cells (Fig. 12). When the PKA/PKC ratio raises to a certain value, cells aggregate to form dermal condensations. When the PKA/PKC ratio decreases to a certain value, cells stop condensing and remain as interbud fibroblasts. Regulation of PKA and PKC in mesenchyme depends on the presence of the epithelium and is probably mediated by some growth factors.

Here we tested the above hypothesis by directly altering PKA and PKC activity intracellularly. The resulting ultralarge merged dermal condensations or the expanded interbud regions are consistent with this hypothesis. In the future, we will search for extracellular signals, growth factors, or adhesion molecules that regulate the reciprocal PKA and PKC activity observed here.

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