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Dissection of the Signaling Mechanism for Capsule Detachment of Lipid Droplets in Rat Adrenocortical Cells

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Abstract In a previous study, we used a monoclonal antibody, A2, to demonstrate the presence of the lipid droplet-specific capsule in adrenocortical cells and the stimulation of steroid secretion with adrenocorticotrophic hormone (ACTH), resulting in the detachment of this capsule from the droplet surface into the cytosol. To investigate the signaling pathway for this event, we tested the role of adenylate cyclase, cAMP, and protein kinases A and C (PKA and PKC) in this response. ACTH-induced decapsulation of lipid droplets was blocked by either adenylate cyclase inhibitor or PKA inhibitor and stimulated by Bt_2cAMP. We conclude that the signaling mechanism involved in lipid droplet decapsulation is the cascade consisting of adenylate cyclase activation, cAMP elevation, and subsequent PKA activation. Furthermore, the cytosolic detached capsular protein was able to relocate to the lipid droplet surface on cessation of ACTH or Bt_2cAMP stimulation. In addition to PKA-mediated decapsulation, inhibition of PKC by calphostin C alone was enough to induce decapsulation, a process that was independent of PKA activity, whereas activation of PKC could prevent Bt_2cAMP-induced decapsulation. A cAMP radioimmunoassay also confirmed that ACTH caused a marked increase in intracellular levels of cAMP, while PMA or calphostin C caused no significant changes. We conclude that PKA and PKC are reciprocally operated to regulate the decapsulation of lipid droplets, the same mechanism adopted in steroidogenesis. A time-course study also indicates that decapsulation of lipid droplets was accompanied by detectable changes in the size and the area of lipid droplets upon the stimulation of Bt_2cAMP or calphostin C, implying a possible coupling between the capsule detachment and steroidogenesis. J. Cell. Biochem. 65:67–74. © 1997 Wiley-Liss, Inc.

Key words: capsule detachment; lipid droplet; adrenal cell; signaling pathway; immunocytochemistry

Autoradiographic study indicates that 70–80% of cholesterol is stored in esterified form within lipid droplets of the rat adrenocortical cells [Moses et al., 1969]. Upon adrenocorticotrophic hormone (ACTH) stimulation, steroid production by adrenocortical cells is increased concomitant with the depletion of the cholesterol pool [Vahouny et al., 1978]. Recent studies have shed some light on the mechanism that causes mobilization of cholesterol from lipid droplets to the mitochondria, one of the major sites for steroidogenesis. The initial stage in the action of ACTH is thought to be the binding to specific cell-surface receptors, which then results in the activation of a membrane-associated adenylate cyclase, turn converting adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) in the cytosol [Grahame-Smith et al., 1967; Rodbell, 1980]. The increased levels of intracellular cAMP activate protein kinase A (PKA), which phosphorylates and activates a number of proteins, including cholesterol ester hydrolase [Beckett and Boyd, 1977; Naghshineh et al., 1978]. In adipocytes, a hormone-sensitive lipase that is similar to cholesterol ester hydrolase in the biochemical property [Cook and Yeaman, 1982] translocates from the cytosol to lipid droplet surface in response to lipolytic stimuli [Egan et al., 1992]. Whether the cholesterol ester hydrolase also behaves so in rat adrenal cells upon ACTH stimulation has not yet been determined.

Recently, perilipins, surface proteins on lipid droplets, are found in adipocytes, Y-1 adrenal

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cells and MA-10 Leydig cells [Servetnick et al., 1995]. Londos et al. [1995] proposed that once hyperphosphorylated by PKA, perilipins may serve as a docking protein on lipid droplet surface for hormone-sensitive lipase. Whether perilipins are also present in rat adrenal cells is unknown.

We have reported a specific monoclonal antibody (mAb), A2, which identifies a 160-kD capsular protein on lipid droplets in rat adrenocortical cells [Wang and Fong, 1995]. Interestingly, the capsular staining of the lipid droplet surface is lost in response to ACTH stimulation [Wang and Fong, 1995]. In the present study, our aims were to elucidate the signal transduction pathway for this ACTH-induced decapsulation of lipid droplets and the relationship between decapsulation of lipid droplets and lipolysis. In addition, since activation of PKC is reported to inhibit ACTH-induced cortisol production [Mason et al., 1986; Ilvesmaki and Voutilainen, 1991], we also examined the role of this enzyme in the process.

MATERIALS AND METHODS

Cell Culture

Primary cultures of rat adrenocortical cells were established as previously described, with a few modifications [Wang and Fong, 1995]. Briefly, isolated cells were obtained by incubating adrenal gland fragments in serum-free M199 medium, containing 1 mg/ml of collagenase (Type II; Sigma, St. Louis, MO), for 30 min at 37°C, with gentle shaking, followed by mechanical dispersion by aspiration using a glass pipette. The dissociated cells were washed, pelleted by low-speed centrifugation, resuspended and cultured in an equi-volume of Ham's F-12 and Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY), supplemented with 1.2 g/L of $NaHCO_3$, 15 mM Hepes, 5% horse serum, 2.5% fetal bovine serum (FBS), 100 IU/ml of penicillin, and streptomycin. Cultures were maintained at 37°C in an atmosphere of 95% air and 5% CO_2 for 2 days.

Drug Treatment

To study the role of adenylate cyclase, 50 μM adenylate cyclase inhibitor, 2′,3′-dideoxyadenosine (DDA) [Londos and Wolff, 1977] was added with or without ACTH at a final concentration of 0.01 IU/ml to adrenal cell cultures for 18 h at 37°C. Adrenal cells were also treated for 1–3 h at 37°C with 0.5 or 1 mM Bt_2cAMP to mimic the effect of cAMP [Kramer et al., 1984]. H-89 (a selective inhibitor of PKA) was administrated to the medium at final concentrations of 250 or 500 nM (5- or 10-fold of K_i for H-89) [Chijiwa et al., 1990] in the presence or absence of 0.5 or 1 mM Bt_2cAMP to examine the involvement of PKA in decapsulation of adrenocortical cells. In signal transduction pathway studies of PKC, either phorbol 12-myristate 13-acetate (PMA), an activator, or calphostin C (an inhibitor) was used at working concentrations of 50 or 100 nM (5- or 10-fold of K_i for PMA) [Castagna et al., 1982], and 250 or 500 nM (5- or 10-fold of K_i for calphostin C) [Kobayashi et al., 1989], respectively. The activity of calphostin C was enhanced by light activation for 1 h [Bruns et al., 1991]. After drug treatment, cells were processed for immunofluorescence staining. ACTH, DDA, and Bt_2cAMP were all purchased from Sigma Chemical Co. H-89, PMA, and calphostin C were purchased from Calbiochem-Novabiochem Int. (San Diego, CA, USA), Gibco, and RBI (Natick, MA, USA), respectively. All experiments were performed with triplicate dishes and more than one hundred cells were examined for each dish. Percentages of decapsulated cells were expressed as mean ±SD.

Immunofluorescence Microscopy

The method used and the preparation of antibodies were as previously described [Wang and Fong, 1995].

Quantitative Measurements

The numbers of lipid droplets in each cell in Figure 5 were counted and the area occupied by total lipid droplets in each cell measured using the BRS-2 version of Microsoft Computer Image Device system (Brock University, St. Catharines, Ontario, Canada).

cAMP Assay

Rat adrenocortical cells (1×10^6 cells/dish) were directly grown in plastic dishes for 2 days. Triplicate culture dishes were treated with ACTH (0.01 IU/ml) for 30 min, PMA (100 nM), and calphostin C (500 nM) for 2 h before the assay. After drug treatment, cells were incubated in physiological salt solution for 20 min at 37°C, followed by 500 nM 3-isobutyl-1-methylxanthine (IBMX) (Sigma) incubation for another 20 min at 37°C, to inhibit phosphodiesterase activity. Cells were then treated with 0.01 N HCl, and the supernatant collected

(15,000 rpm × 5 min) and neutralized with 1 N NaOH for cAMP assay. Intracellular cAMP level was measured according to the radioimmunoassay (RIA) methods of the Amersham cAMP [^{3}H] assay system (Amersham, Buckinghamshire, UK).

RESULTS

The adrenal cells were found to contain a number of lipid droplets. At the light microscopic level, immunofluorescence staining with antibody A2 demonstrated the capsule around the lipid droplet to be complete (Fig. 1A).

ACTH treatment caused the fluorescent staining to move from the lipid droplet surface into the cytosol of adrenal cells (Fig. 1B, control Fig. 1A). This translocation of capsular staining was observed in 45 ± 2.5% of the adrenal cells. Treatment with DDA alone produced no change in the appearance of the capsule compared with the normal control (Fig. 1C). However, when added at the same time as ACTH, the capsule remained intact (Fig. 1D), indicating that the cAMP pathway is essential in the process. We then applied Bt_2cAMP to the cultures in order to mimic the effect of cAMP. As expected, decapsulation occurred in many adrenal cells within 1–3 h, the extent of which was time and concentration dependent. The fluorescent staining of the capsule became discontinuous 1 h after Bt_2cAMP application (Fig. 2A), then gradually diminished up to 3 h of treatment (Fig. 2B), whereas cytosolic staining increased concomitantly. About 73.7 ± 5.7% and 80.8 ± 7.7% of the adrenal cells contained decapsulated lipid droplets when treated with 0.5 or 1 mM Bt_2cAMP for 3 h, respectively. The changes from capsular staining to amorphous cytosolic staining was reversible; one h after the replacement of 1 mM Bt_2cAMP with fresh control medium strong staining of the capsule on the remaining lipid droplets was seen (Fig. 2C). Similar reversibility of the effect was seen when using ACTH (Fig. 2D).

Lipid droplets devoid of capsule staining were found in 73.6 ± 5.2% of the adrenal cells when treated with 1 mM Bt_2cAMP alone for 2 h (Fig. 3A). Treatment with 500 nM H-89 alone did not cause any change in the capsular staining. When H-89 (500 nM) was added in combination with 1 mM Bt_2cAMP, the capsule of lipid droplets in most adrenal cells remained highly fluo-

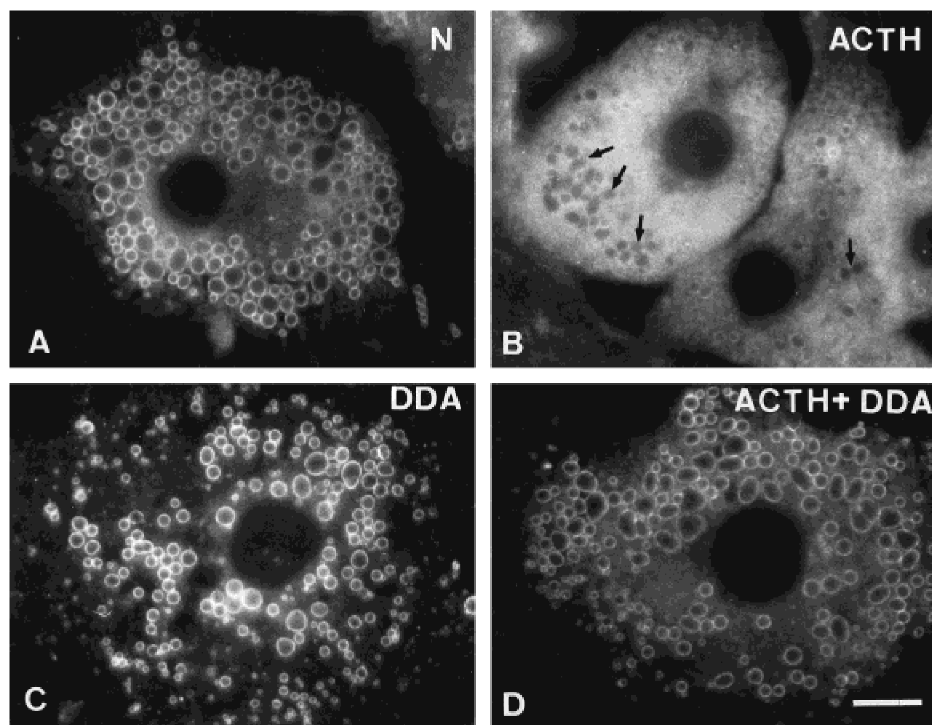

Fig. 1. Involvement of adenylate cyclase in the decapsulation of lipid droplets. **A:** Untreated normal control. All lipid droplets are enclosed by a bright fluorescent capsule. **B:** At 18 h after ACTH stimulation, the surface of many lipid droplets is not immunostained (*arrows*). **C:** At 18 h after DDA application, capsular staining is intact and bright. **D:** At 18 h after the simultaneous application of ACTH and DDA, bright fluorescence is seen around the surface of the lipid droplets. Bar = 10 μm.

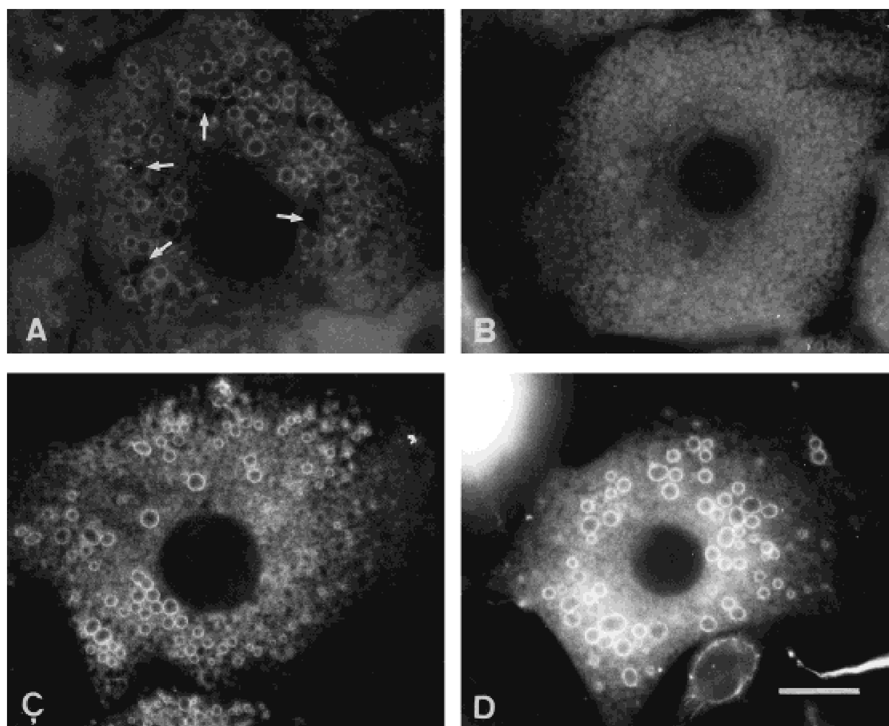

Fig. 2. Time-dependent effect of Bt_2cAMP on the capsular staining of lipid droplets. **A:** At 1 h after 1 mM Bt_2cAMP treatment, capsular staining is incomplete or discontinuous (*arrows*). **B:** At 3 h after 1 mM Bt_2cAMP treatment, the size and number of lipid droplets are reduced, capsular staining has been lost, and the cytosol is filled with a fluorescent amorphous substance. **C:** Cells treated with 1 mM Bt_2cAMP for 3 h, then transferred to fresh medium for 1 h. Although the cytosol is still filled with an amorphous substance, capsular staining on some lipid droplets is evident. **D:** Cells treated with 0.01 IU ACTH for 18 h, then transferred to fresh medium for 1 h. The lipid droplets are surrounded by a prominent fluorescent capsule. Bar = 10 μm.

rescent and intact (Fig. 3B). This finding suggests that activation of PKA is required for the Bt_2cAMP-induced decapsulation of lipid droplets. The present study therefore demonstrates that the signal for capsular protein detachment is transduced by a cascade consisting of ACTH, cAMP, and PKA.

To test the role of PKC in regulating decapsulation of lipid droplets, the activities of PKC, which were monitored by specific activator or inhibitor, were correlated with the staining pattern of antibody A2. Treatment with 50 or 100 nM PMA alone had no effect on the capsular staining of lipid droplets (Fig. 3C), whereas simultaneous activation of PKC greatly inhibited Bt_2cAMP-induced decapsulation of lipid droplets in adrenal cells (Fig. 3D). By contrast, when PKC activity was inhibited by 250 or 500 nM calphostin C, the decapsulation of lipid droplets occurred in 74.5 ± 1.4% or 91.9 ± 5.1% of the adrenal cells, respectively (Fig. 3E). In order to determine whether this decapsulation caused by PKC inhibition is related to PKA activity, H-89, added either before or at the same time as calphostin C to the cultures, failed to prevent the decapsulation of lipid droplets in the adrenal cells (Fig. 3F). These data suggest that PKA is not required in calphostin C-induced decapsulation.

A cAMP RIA indicated that stimulating adrenal cells with ACTH increased the intracellular cAMP level by about 22-fold (59.8 ± 5.03 pmole/mg protein, compared with 2.64 ± 0.24 pmole/mg protein in controls) (Fig. 4). By contrast, neither PMA nor calphostin C caused any significant change in cAMP levels. These data also support the idea that the calphostin C-mediated decapsulation of lipid droplets does not involve an increase in intracellular cAMP levels.

To determine whether lipid droplet decapsulation leads to hydrolysis of the droplet, we performed time-course recording of droplet morphology in the presence of various stimuli, then carried out immunostaining on the same cultures. In normal adrenal cells, the distribution

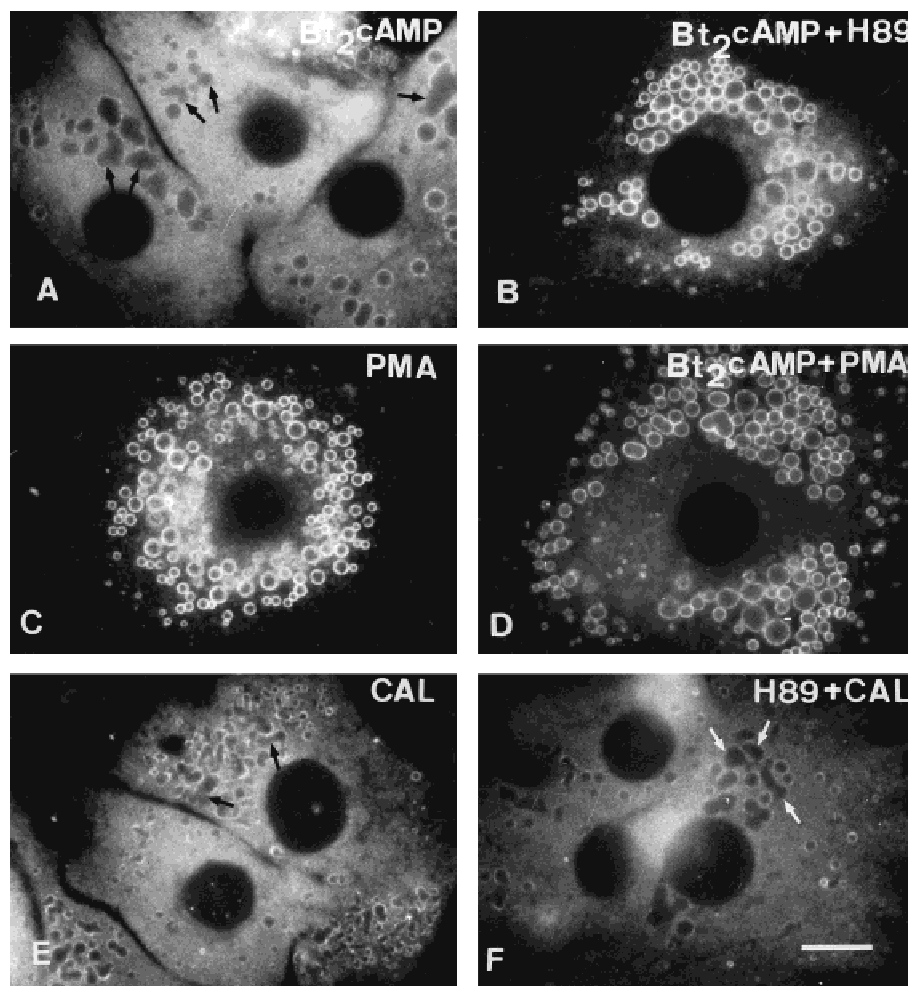

Fig. 3. Involvement of PKA and PKC in decapsulation of lipid droplets. **A:** At 2 h after application of 1 mM Bt_2cAMP, the staining intensity of the capsules appears to be reduced on these polymorphic lipid droplets (*arrows*). **B:** At 2 h after application of 1 mM Bt_2cAMP plus 500 nM H-89, the staining on the lipid droplet surface is strong and continuous. **C:** At 2 h after application with 100 nM PMA, the capsules of the lipid droplets appear normal. **D:** At 2 h after application of 1 mM Bt_2cAMP plus 100 nM PMA, staining of the lipid droplets is continuous and strong. **E:** At 2 h after application of 500 nM calphostin C, the capsules are incomplete and irregular in contour (*arrows*). **F:** Cells treated with H-89 for 2 h and then with calphostin C for another 2 h. Antibody-labeled capsule is detached from lipid droplets (*arrows*). Bar = 10 μm.

of lipid droplets in the cytoplasm was dynamic; however, the number and area of the droplets remained constant. The control cell shown in Figure 5A contained 142 lipid droplets (total area 137.3 μm^2) at the beginning of observation and 152 lipid droplets (total area 133.4 μm^2) at the end of an 8-h period. All lipid droplets in control cells were surrounded by a bright continuous capsule (Fig. 5D). In Figure 5B, the number of lipid droplets in Bt_2cAMP-treated cells at the beginning and at the end of the 8-h Bt_2cAMP treatment was 73 and 61, respectively, those remaining being the smaller droplets. The total area was significantly decreased by 36% from 115.6 μm^2 to 74.3 μm^2. The fluorescent labeling on these remaining lipid droplets was discontinuous and weak compared with that of normal control cells (Fig. 5E). More marked changes in lipid droplets morphology and cell shape were seen with calphostin C treatment (Fig. 5C). Initially, the lipid droplets were round, 229 in number and with an area of 137.6 μm^2; after 8 h of calphostin C treatment, they appeared to be fused and were irregular in shape, their numbers dropping to 102 and the total area decreasing by 18% to 113.4 μm^2. The cells became retracted, and cytoplasmic movement of lipid droplets ceased. In addition, most of the lipid droplets were not immunostained; instead, prominent amorphous staining was

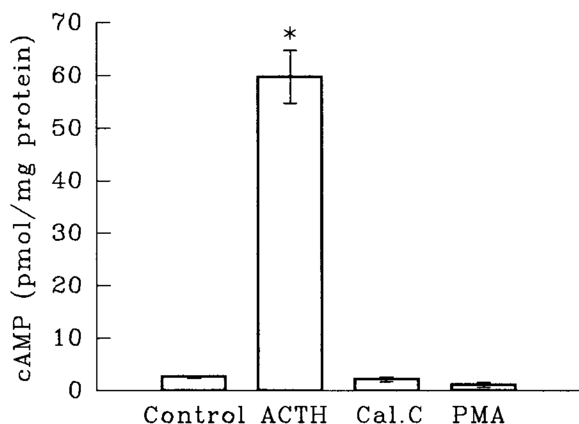

Fig. 4. Effects of various reagents on intracellular levels of cAMP. Adrenal cultures were pretreated with ACTH, calphostin C, or PMA prior to assay. The values are expressed as the mean ±SD of triplicate determinations. The asterisk indicates a significant increase of cAMP content in ACTH-treated cells compared with that in control (* $P < 0.05$).

seen in the cytosol (Fig. 5F). This experiment indicates that lipid droplet decapsulation induced by Bt_2cAMP or calphostin C is accompanied by varying degrees of lipid droplet hydrolysis.

DISCUSSION

In our previous studies, immunofluorescence experiments demonstrated that, in both cultured rat adrenal cells [Wang and Fong, 1995] and in cultured hamster Leydig cells [Fong et al., 1996], individual lipid droplets are surrounded by a capsule, which may act as a protective protein barrier, preventing lipid droplet hydrolysis in unstimulated cells. Lipid droplets in both cell types were decapsulated following stimulation of the adrenal cells by ACTH or the Leydig cells by LH. The present study shows that the detached proteins in the cytosol of adrenal cells are able to redistribute to the lipid droplet surface following termination of ACTH stimulation, suggesting that the conformational changes induced in the protein are both transient and reversible.

The significance of lipid droplet decapsulation was explored by time-lapse observations of morphological changes in the droplets following stimulation with calphostin C or Bt_2cAMP; the experiment showed the lipid droplets which decrease in size or fuse to be those which had become decapsulated. The capsule may therefore protect the lipid droplet in the unstimulated cell from hydrolysis or fusion. Since activation of cholesterol ester hydrolase (CEH) [Beckett and Boyd, 1977] and decapsulation of lipid droplets (present study) are both turned on by PKA, decapsulation of the lipid droplet may allow the activated CEH direct access to its substrate, thus accelerating lipolysis. This is the situation seen in isoproterenol-stimulated 3T3-L1 adipocytes, in which the hormone-sensitive lipase is translocated from the cytoplasm to the lipid droplet surface [Egan et al., 1992; Londos et al., 1995]. Moreover, since most adrenal cholesterol is contained within lipid droplets [Moses et al., 1969], their dissolution would result in an increase in the cytosolic cholesterol pool, which is then available for steroid production in the mitochondria and smooth endoplasmic reticulum.

Perilipins are known to be involved in lipid packing and hydrolysis in adipocytes [Londos et al., 1995]; however, they do not translocate in response to lipolytic stimulation [Servetnick et al., 1995; Londos et al., 1995]. Unlike the situation with the perilipins, it is possible that the 160-kDa protein must first be detached from the surface of the lipid droplet, thus allowing the CEH access to the lipid in the droplet. Currently, we are attempting to investigate the temporal and spatial relationship between decapsulation and the translocation of CEH.

The signal transduction pathway responsible for capsule detachment was elucidated by examination of the changes in capsular morphology of lipid droplets in adrenal cells treated with specific inhibitors or drug analogues and was found to involve activation of adenylate cyclase, increased cAMP levels, and activation of PKA, that is, the same pathway involved in ACTH-induced steroidogenesis in adrenal cells [Simpson and Waterman, 1988]. We also found that the PKA-mediated decapsulation was significantly blocked by PKC activation, indicating a negative regulatory role for PKC in decapsulation. Furthermore, inhibition of PKC activity by calphostin C also caused decapsulation, again supporting the idea that PKC may be responsible for suppressing decapsulation in rat adrenocortical cells. The pathway involved in lipid droplet decapsulation induced by PKC inhibition is as yet unidentified, but it clearly does not involve the PKA system, as it was not blocked by H-89 pretreatment and PKC inhibition did not change the levels of intracellular cAMP. Positive and negative control in steroido-

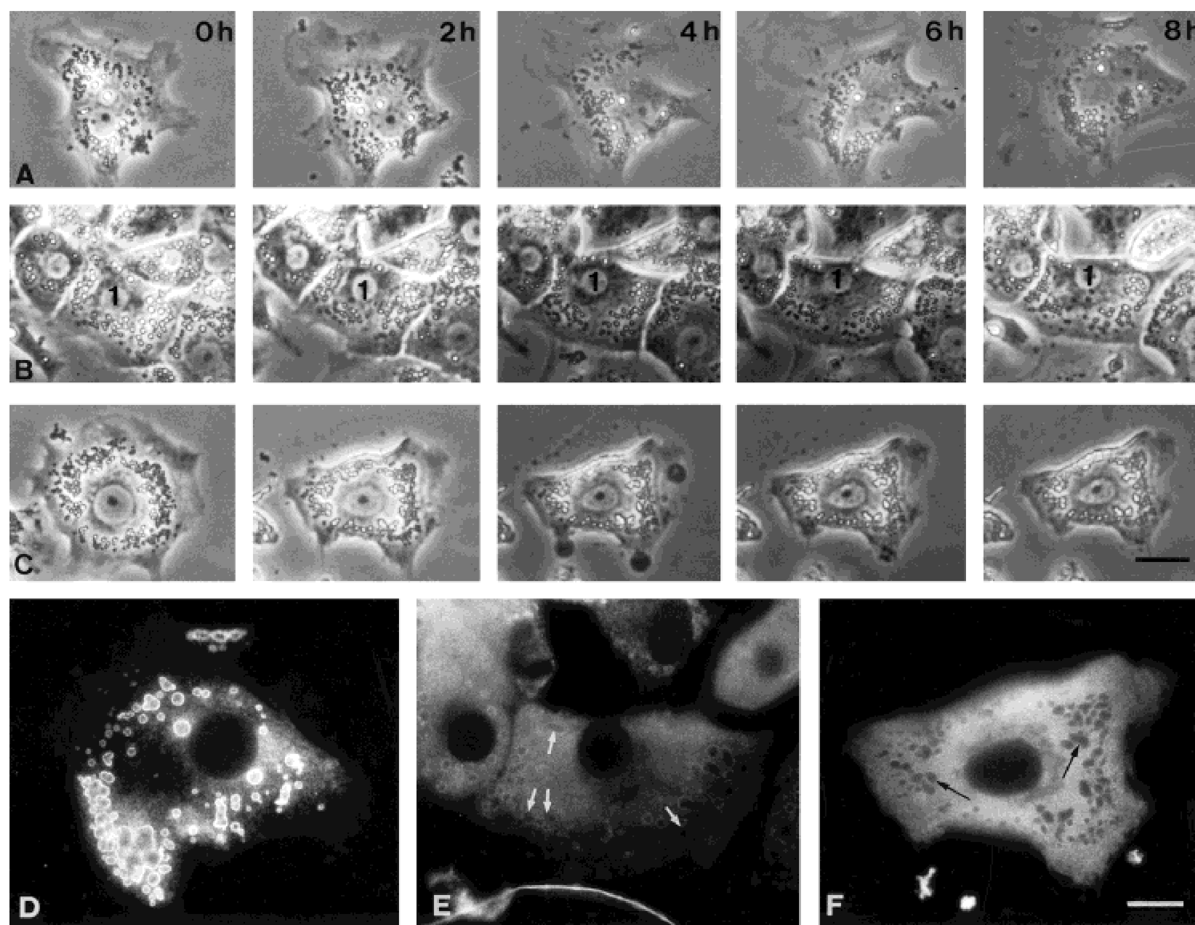

Fig. 5. A time-course study on lipid droplets in adrenal cells treated with Bt_2cAMP and calphostin C treatments. **A:** Control cells. Although lipid droplets change their relative position in the cytoplasm, their size and number remain the same in the total period of 8 h. **B:** Cells incubated with 1 mM Bt_2cAMP. A decrease in the number and size of lipid droplets is seen in cell (1) 2–8 h after treatment. **C:** Cells incubated with 500 nM calphostin C. The changes in the shape and number of lipid droplets are obvious at 2 h after treatment. **D–F:** Immunostaining with antibody A2 on the same cells from A, B, and C groups, respectively. Lipid droplets in control cells have a fluorescent capsule (**D**). After treatment with Bt_2cAMP (**E**) or calphostin C (**F**), the capsules are detached from the lipid droplets (*arrows*). **A–C:** Bar = 20 μm. **D–F:** Bar = 10 μm.

genesis by PKA and PKC, respectively, is well established in the mouse adrenal Y-1 cell [Reyland, 1993]. Our hypothesis that lipid droplet decapsulation is coupled to steroidogenesis is supported by the similar signal transduction pathway shared by these two events.

Many PKC isoforms have been identified in mammalian tissues [Nishizuka, 1992]. Since bovine and rat adrenocortical cells express only the α isoform of PKC [Pelosin et al., 1991], it is possible that this subtype mediates the regulation of decapsulation of lipid droplets, although further study is required to verify this hypothesis. The mechanism as to how the inhibition of PKC leads to the capsule detachment remains to be studied.

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