Effect of okadaic acid on utilization of lipoprotein-derived cholesteryl esters by rat steroidogenic cells

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Abstract This study examines various functional, biochemical, and structural changes in rat adrenocortical and ovarian granulosa cells that could account for the decline in lipoprotein-supported hormone production after cell treatment with the protein phosphatase inhibitor, okadaic acid. Although the steroidogenic pathway enzymes in these cells are not in themselves affected by okadaic acid, the intracellular transport of cholesterol to important cellular processing sites is defective. That is, okadaic acid does not interfere with the internalization of lipoprotein-derived cholesteryl esters, but the mitochondrial utilization of cholesterol obtained from intracellular cholesterol storage sites is 50% reduced as compared to control cells. Two-dimensional electrophoresis gels from okadaic acid-treated cells demonstrate a number of hyperphosphorylated proteins. Morphological examination of the affected cells reveal completely disrupted Golgi complexes with attendant structures, but otherwise the cells appear unchanged. The results suggest that some necessary sterol transport protein (or cofactor or associated membrane) is adversely phosphorylated by okadaic acid, and is rendered dysfunctional.—**Azhar, S., J. A. Frazier, L. Tsai, and E. Reaven.** Effect of okadaic acid on utilization of lipoprotein-derived cholesteryl esters by rat steroidogenic cells. *J. Lipid Res.* 1994. **35:** 1161–1176.

Supplementary key words cholesteryl ester transport • rat adrenal and granulosa cells • Golgi disruption • steroidogenesis • protein phosphorylation

We have previously shown that okadaic acid (OKA), a potent inhibitor of serine/threonine-specific protein phosphatases 1 and 2A (1, 2), dramatically reduces lipoprotein-induced corticosterone production in cultured adrenocortical cells (3). Under the conditions of our experiments, adrenocortical cells normally respond to stimulation with their tropic hormone, ACTH, and a variety of other stimulatory factors (e.g., Bt_2 cAMP, 8 BrcAMP, cholera toxin) with a 10- to 15-fold rise in corticosterone production over basal levels. With added lipoproteins as an exogeneous source of cholesterol, the corticosterone response increases an additional 25-fold.

With the addition of OKA (100 nM) to the cultures, this expected 250- to 400-fold lipoprotein-induced rise in steroidogenesis is almost totally blocked. The OKA effect is both time- and concentration-dependent. It is not related to cell surface or trophic hormone receptor function as the response to ACTH, or any of the other stimulating agents alone, is not affected. Indeed, the lack of an OKA effect on cells treated with these stimulating agents suggests that the enzymes of the steroidogenic pathway are not affected. The response seen after OKA treatment appears specific for the cellular uptake and/or transport of lipoprotein-derived cholesterol to mitochondrial sites where cholesterol side-chain cleavage occurs as the first step in steroidogenesis. The rationale for this thinking is that while OKA prevents the lipoprotein-induced rise in adrenocortical cell corticosterone synthesis from a variety of lipoprotein groups tested (including human and rat HDL, LDL, and various modified lipoproteins), it does not affect the rise in hormone production when the cells are given 20 α-hydroxycholesterol, a compound that is freely diffusible in cells, and does not require active transport mechanisms (4–6).

In the current report, we examine this issue further in cells from the rat adrenal cortex and ovary. We determine that OKA does, in fact, specifically affect serine/threonine type phosphatases in steroidogenic cells. It does not alter the internalization of lipoprotein-derived cholesteryl esters by either the endocytic (7, 8) or "selective" (9–14) pathways in these cells, but it has a major impact on the utilization of newly acquired cholesterol by mitochondria. It appears that a specific intracellular target of OKA is the Golgi complex, and we attempted to track the dynamics of OKA-induced Golgi membrane breakdown with changes in lipoprotein-derived cholesteryl ester utilization.

Abbreviations: OKA, okadaic acid; HDL, high density lipoprotein; LDL, low density lipoprotein; CLE, cholesteryl linoleyl ether.

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MATERIALS AND METHODS

Materials

[^{3}H]cholesteryl oleate (sp act 2.22–3.70 TBq/mmol, 60–100 Ci/mmol) and Na^{125}I (carrier-free) (sp act 643.8 GBq/mg; 17.40 Ci/mg) were purchased from E. I. DuPont de Nemours and Co. (Wilmington, DE). [1α, 2α (n)-^{3}H]cholesteryl linoleyl ether ([^{3}H]CLE), sp act 1.1–2.2 TBq/mmol, 30–60 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Human plasma transferrin and human plasma fibronectin were the products of Collaborative Research (Bedford, MA). Okadaic acid, calyculin A, 1-nor-okadaone, methyl okadadate, okadol, and okadaic acid tetracetate were supplied by LC Services Corp. (Woburn, MA). Corticotropin, a synthetic subunit of ACTH (Cortrosyn) was obtained from Organon Inc. (West Orange, NJ). Brefeldin A was purchased from Epicenter Technologies (Madison, WI). All other reagents used were of analytical grade.

Cells isolation and culture conditions

Adrenocortical cells were used for the majority of studies. Control or 4 aminopyrazolo [3,4-d]pyrimidine (4-APP, 20 mg/kg body weight, i.p. every 24 h for 3 days) -pretreated male Sprague-Dawley rats (200–240 g, Bantin and Kingman, CA) were used as cell donors; 4-APP treatment reduced circulating levels of lipoproteins and adrenal cholesteryl ester content (13). These cells were isolated and cultured as previously described from this laboratory (3). Unless otherwise noted, the cells were maintained in DME:F12 medium containing 1% lipoprotein-deficient calf serum for 24 h before experiments with additives commenced.

Granulosa cells were obtained from the ovaries of immature rats primed for 5 days with 17β estradiol by methodology described from this laboratory (13). The cells were cultured for 72 h in serum-free medium before the addition of other agents.

At the conclusion of all biochemical experiments, the incubation medium was centrifuged to recover floating cells. These cells were processed along with the attached cells.

Lipoprotein preparation

Lipoproteins were prepared and/or modified as previously published (3, 11–13).

Phosphorylation of cell proteins

Cultured adrenal cells were incubated with [^{32}P]Pi (0.35 mCi/ml) ± OKA (100 nM) in a phosphate-free medium at 37°C. After incubation (60–120 min), the cells were rapidly washed (3 times), resuspended in isoelectric focusing buffer, and sonicated cell homogenates were immediately heated at 95–100°C for 2 min and then subjected to two-dimensional (2D) gel electrophoresis by the method of O'Farrell (15) with minor modifications (16). The 2D gels were stained with silver by a modification (17) of the method of Merril et al. (18) and subjected to autoradiography. Autoradiography was carried out in cassettes with Kodak X-O-mat XAR-5 film using a Dupont Cronex Lighting Plus enhancing screen. Films were exposed for 5–10 days at −80°C before being developed.

To rule out the possibility that phosphotyrosine residues are among the hyperphosphorylated proteins in OKA-treated adrenals, samples were subjected to one-dimension SDS-PAGE and transferred to Immunobilon membranes. Subsequently, membrane blots were incubated first with phosphotyrosine antibodies (Zymed Labs, Burlingame, CA) then developed with alkaline phosphatase-conjugated goat antibody to rabbit immunoglobulin G, 5 bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium. Blots from control and OKA-treated cells were scanned for changes in phosphotyrosine using an LKB laser densitometer.

Steroidogenesis

To assay steroidogenesis, cultured adrenal cells were pre-treated with or without OKA (100 nM) for 30 min. Subsequently, triplicate culture dishes were supplemented with ACTH (10 ng/ml) or Bt_2 cAMP (2.5 mM) ± $hHDL_3$ (500 μg protein/ml), rHDL (100 μg protein/ml), or hLDL (100 μg protein/ml). After incubation at 37°C for 3, 6, or 24 h, samples of incubation medium were frozen and stored until assayed for corticosterone. Corticosterone was assayed by direct radioimmunoassay (RIA) using specific antiserum as described previously (3). Results are expressed as pmol corticosterone produced per μg cellular DNA. Results presented are mean ± SE of duplicate determinations of three different dishes.

The incubation conditions for granulosa cell steroidogenesis were similar except ACTH was replaced with FSH (50 ng/ml) (13). At the end of each incubation, samples of incubation medium were frozen, and stored until assayed for progestins. Progesterone and its metabolite 20 α-dihydroxyprogesterone were quantified by RIA using specific antiserum as described previously (13). Results are expressed as pmol progestin (the sum of progesterone and 20 α-dihydroxyprogesterone) produced/μg DNA and represent the mean ± SE of duplicate determinations of three different dishes.

Measurement of lipoprotein-derived cholesteryl ester incorporation into corticosterone

Incorporation of $hHDL_3$-, rHDL-, or hLDL-derived [^{3}H]cholesteryl ester into cortiocosterone and other sterols was measured as described previously (19). Briefly, cultured adrenal cells were incubated in 1.5 ml medium DME:F12 containing 1% lipoprotein-deficient serum, 100 μg protein/ml [^{3}H]cholesteryl ester $hHDL_3$, rHDL or hLDL ± OKA (100 nM) and ACTH (10 ng/ml). After incubation at 37°C for 3 or 6 h, the incubation media

were removed and saved. The cells were washed extensively with culture medium containing 0.5% BSA to remove any absorbed extracellular radioactivity. Suitable aliquots of incubation medium and cells were extracted and quantified for [^{3}H]corticosterone, [^{3}H]cholesterol, and [^{3}H]cholesteryl esters (19) and mass of the corticosterone produced (20). The results are expressed either as pmole of cholesteryl ester incorporated/μg DNA or as pmole of mass of corticosterone synthesized. In each case, greater than 99% of the corticosterone produced had been secreted into the incubation medium.

Cholesteryl ester internalization by cells

In these experiments medium from 24-h cultured cells was replaced with fresh medium containing ACTH ± OKA and $hHDL_3$, rHDL, or hLDL, which had been equipped with radiolabeled, non-releasable apoprotein and cholesteryl ester tags that would accumulate within the cells even when degraded (12, 13). Incubations were carried out with 50 μg protein/ml ^{125}I-labeled dilactitol-[^{3}H]cholesteryl linoleyl ether ([^{125}I] DLT-[^{3}H]CLE) lipoprotein + ACTH (10 ng/ml) ± OKA (100 nM) for 3, 6, and 24 h at 37°C. At the end of incubation, the accumulated cells were washed four times with PBS–0.1% bovine serum albumin, once with PBS, and subsequently solubilized in 2 ml of 0.1 M NaOH. One-ml aliquots were precipitated with an equal volume of 20% trichloroacetic acid to determine insoluble and soluble radioactivities (12, 13) or extracted with organic solvents (12, 13) to determine ^{3}H radioactivity.

Under the conditions used, trichloroacetic acid-insoluble ^{125}I radioactivity was assumed to represent ^{125}I-labeled protein remaining bound to the cell surface as part of intact lipoproteins (12, 13); trichloroacetic acid-soluble ^{125}I radioactivity, was taken to be internalized, degraded, and accumulated residualizing protein ^{125}I label. As the ^{125}I and ^{3}H labels are on the same lipoprotein particles, it follows that the relative amounts of surface bound ^{125}I and ^{3}H radioactivity must be equal. Thus, the amount of cholesteryl ester internalized can be computed as the difference between total cholesteryl ester uptake and trichloracetic acid-insoluble (i.e., surface-bound) ^{125}I radioactivity. The results are expressed as pmole ^{125}I- or ^{3}H-labeled protein internalized/μg DNA. To determine the net mass of cholesteryl ester internalized, ^{3}H protein values are divided by the protein to cholesterol ratio of each lipoprotein (e.g., for $hHDL_3$, rHDL, or hLDL the respective protein/cholesterol ratios are 2.44, 1.10, and 0.55.

Mitochondrial side-chain cleavage enzyme activity

Cultured adrenal cells (5×10^7) obtained from control (non-4APP) rats were treated with ACTH (10 ng/ml) ± OKA (500 nM) for 3 h at 37°C. The cell preparations were next homogenized and mitochondria were isolated by a "hybrid" Percoll/Metrizamide discontinuous gradient centrifugation procedure as described by Madden and Storrie (21). The isolated mitochondrial fractions were used to measure cholesterol side-chain cleavage activity (P450scc) in vitro (22) using pregnenolone production as the assay. Two types of studies were conducted. In the first, the substrate for the assay was the cholesterol that the mitochondria had accumulated during the 3-h cell incubation with (or without) OKA. But, because the effect of OKA was found to reduce the pregnenolone produced by these mitochondria, it was necessary also to evaluate the impact of OKA on the P450scc enzyme system per se. This was accomplished in the second study by supplying mitochondria with a freely diffusible form of cholesterol (i.e., 20α-hydroxycholesterol); this cholesterol equalized available mitochondrial substrate permitting an independent assay of mitochondrial P450scc activity. The incubation medium contained 0.25 M sucrose, 10 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 10 mM potassium phosphate, 25 mM Tris-HCl (pH 7.4), 10 μM cyanoketone, 1 mg/ml fatty acid-free BSA, and 50 μg/ml mitochondrial protein in the presence or absence of 25 μg/ml 20α-hydroxycholesterol. After incubation at 37°C for 1.0–10 min, the reaction was terminated by quick freezing at −60°C. The reaction product, pregnenolone, was extracted from the incubation with hexane and assayed by specific RIA. Cyanoketone was included to inhibit further metabolism of pregnenolone to pregesterone (22). All assays were run in triplicate and enzyme activity was expressed as pmole pregnenolone formed/min per mg protein.

Morphological techniques

Immunofluorescence microscopy. The following antibodies were used: monoclonal mouse anti-58 kDa protein (23), a gift from Dr. George Bloom (University of Texas Southwestern); monoclonal mouse anti-βCOP (110 kDa protein (24), a gift from Drs. Duden and T. Kreis (Heidelberg, Germany); affinity-purified second antibodies including FITC-labeled goat anti-mouse IgG (Tago, Inc.). The primary antibodies were used at a dilution of 1:200 for 2 h at 37°C. The fluorescent second antibody was diluted 1:100 and used for 30 min. at 37°C. All antibodies were diluted in PBS containing 1% BSA.

Cells growing on no. 1 coverslips were washed twice with PBS, then fixed and permeabilized by a 5-min incubation in −20°C absolute methanol followed by a PBS wash and a dip into PBS + 1% BSA. After incubation with the first antibody as described above, the coverslips were washed 3 times for 3 min each in PBS, dipped into PBS + 1% BSA, and incubated with the fluorescently labeled second antibody. The coverslips were again washed 3 times for 3 min each, dipped into deionized water, and mounted onto glass slides with fluormount (Fisher Scientific, Pittsburgh, PA). All PBS washes were carried out at room temperature. The specimens were viewed

with a Leitz Orthoplan 2 microscope using a 63X planapo objective, and photographed on Kodak T MAX 400 film, push processed to 1600.

Electron microscopy. Cells maintained in 60-mm dishes were washed, fixed for 10 min in their dishes with 1% glutaraldehyde, scraped with a plastic spatula, suspended in a microfuge tube, and spun for 30 sec at 10,000 *g*. The resulting cell pellets were left in fixative overnight and then processed by standard techniques (11, 25–28).

Quantification of microtubules (i.e., accumulated length of microtubules associated with the Golgi region) were carried out as follows. Thin sections were prepared of control and OKA-treated cells. Typically, we examined two blocks each of three separate experiments representing different time points. The first ten cells (with nuclei and some semblance of a Golgi complex) encountered in each thin section were photographed at 14,000×. The micrographs were photographically enlarged 3×, Golgi regions were outlined, and their cytoplasmic volume density was estimated using an image analysis system (Bioquant II, R and M Biometrics, Nashville, TN). Microtubule lengths that fell within these outlined Golgi areas, or within a 1-inch border of these regions on the enlarged photographs (at a final magnification of 54,000×), were measured and expressed as microtubule length per unit Golgi area. Golgi regions included areas with stacked membranes, vesicles, closely associated membrane-bound vacuoles, and occasionally centrioles. The determination of Golgi regions in OKA-treated cells was usually made by tell-tale clusters of vesicles at 3 h.

Miscellaneous techniques

Neutral cytosolic cholesteryl esterase activity was determined using the established procedure of this laboratory (6, 29). Total protein synthesis was measured by following incorporation of [^{35}S]methionine into cellular protein as described previously (30). Cellular ATP and lactate dehydrogenase activities were measured according to the procedures of McCall et al. (31) and Jauregui et al. (32). The DNA content of the cells was quantified fluorometrically (33). The procedure of Markwell et al. (34) was used to quantify protein content of cytosolic and mitochondrial fractions.

RESULTS

Cell characteristics

Isolated adrenocortical cells (from control or plasma cholesterol-depleted rats) grown in culture for 24 h retain most of the intracellular morphological characteristics (lipid droplets, extensive smooth endoplasmic reticulum, mitochondria with vesicular cristae) associated with cells from the Z. fasciculata of the adrenal. Their only apparent structural change is the loss of surface microvilli, a function of the collagenase isolation procedure. Despite this, trophic hormone-stimulated adrenocortical cells are very responsive to cholesterol-rich lipoproteins (which are in continuous contact with the cell surface during static incubations), and will secrete corticosterone at 25 times the levels seen with ACTH alone. It is of interest that both the "endocytic" and "selective" pathways for lipoprotein-cholesterol uptake are operative in the cultured adrenocortical cells [in contrast to the behavior of these cells in situ (12)], and the extent to which each pathway is used is dependent entirely on which lipoprotein is presented to the cells. Thus, adrenocortical cells secrete virtually similar amounts of corticosterone when given human low density lipoproteins (hLDL) or rat high density lipoproteins (rHDL) which bind to the BE receptor (7, 8), or $hHDL_3$, or methylated, or cyclohexanedione-modified hLDL which do not bind to the BE receptor but deliver their cholesteryl ester via the "selective" pathway without internalization of the intact lipoprotein particle (3, 11–14). In these cells, ACTH does not by itself change lipoprotein cholesteryl ester uptake by the "selective" pathway, and only minimally affects (~15%) cholesteryl ester uptake by the endocytic pathway. For convenience, therefore, most of the studies in this report were carried out on trophic hormone-stimulated cells, and for the most part, the cells were provided $hHDL_3$ as their cholesterol source.

The rat-derived ovarian granulosa cells used for these studies have been described previously from this laboratory (13), and like the adrenocortical cells, respond to a variety of cholesterol-rich lipoproteins and hormones (Bt_2cAMP or FSH) with extremely high levels of secreted hormone (13).

After treatment with OKA [10–100 nM], we noticed that the cells underwent shape alterations that were time- and dose-related and which corresponded roughly with intracellular morphological and functional changes observed in the same cells. In general, both adrenocortical and granulosa cells round up after OKA treatment, and if the dose or time is extended, a proportion of the cells will detach.

With adrenal cells (which do not undergo cell division in culture), it was possible to evaluate recovery from the OKA effect. Cells were pretreated with a low concentration of OKA (50 nM) for 3 h (or until the large majority of the cells were rounded but remained attached), after which the cells were thoroughly washed and recultured up to 24 h. Reflattening started slowly around 3 h, but was not complete until some time between the 6- to 24-hour time points. Thus, the very same cells that had been affected by OKA could reflatten with time, but the process was slow.

Specificity and toxicity of OKA effect

Treatment of cells with 100 nM OKA for up to 6 h does not result in deterioration of protein synthesis, ATP

TABLE 1. Adrenal cell ATP content, protein synthesis, and release of lactic dehydrogenase

Measurements	Incubation Time	Control	OKA
	h		*100 nM*
ATP (nmol/mg protein)	3	2.10 ± 0.32	2.00 ± 0.26
	6	2.17 ± 0.61	2.20 ± 0.25
Protein synthesis (CPM × 10^{-3}/μg DNA)]	3	439 ± 103	734 ± 73
	6	647 ± 128	701 ± 132
Lactic dehydrogenase (mU/μg DNA ± SE)	3	91 ± 6	102 ± 16
	6	81 ± 10	95 ± 14

Results are mean ± SE of three separate experiments.

levels, or release of lactate dehydrogenase activity (**Table 1**). Treatment with OKA tetraacetate (50 nM), a nonactive OKA analog, has no apparent morphological or functional effect on adrenocortical or granulosa cells, although OKA itself and other similar inhibitors of protein dephosphorylation (e.g., Calyculin A) dramatically reduce steroidogenesis (**Table 2**). Likewise, brefeldin A, which is known to attack Golgi membrane coat proteins (24, 35, 37), interferes with steroid hormone production (Table 2).

^{32}P phosphorylation of cellular proteins

For these experiments, adrenocortical cells were preincubated with ^{32}P-labeled Pi ± OKA (100 nM) for 120 min before homogenization and 2D gel electrophoresis. **Figs. 1A and 1B** show that OKA treatment generally increases the phosphorylation of adrenal cell phosphoproteins. Several lower molecular weight proteins (between 40- and 10 kDa) were particularly well resolved, and in most instances (arrows 1–4, 6) phosphorylation was enhanced by OKA; in one notable protein (arrow 5), OKA treatment was associated with less phosphorylation. Control experiments showed that OKA did not affect the phosphorylation of tyrosine residues in the same specimens (data not shown).

TABLE 2. Effect of okadaic acid, okadaic acid analog, brefeldin A or calyculin A on corticosterone production

Additions	Amount	Corticosterone
		pmol/μg DNA ± SE
Experiment 1		
None		6053 ± 146
Brefeldin A	250 nM	2715 ± 55
Brefeldin A	1000 nM	788 ± 90
Okadaic acid	100 nM	577 ± 45
Experiment 2		
None		5106 ± 617
Calyculin A	0.2 nM	4152 ± 124
Calyculin A	1.0 nM	427 ± 46
Okadaic acid	100 nM	620 ± 79
Experiment 3		
None		4211 ± 189
Okadaic acid	100 nM	463 ± 47
Okadaic acid tetra acetate	100 nM	4288 ± 547

Cultured adrenocortical cells were incubated with ACTH + $hHDL_3$ ± indicated concentrations of brefeldin A, calyculin A, or OKA analog for 24 h. Secreted corticosterone was measured by RIA. The results are mean ± SE of three separate experiments.

Cell ultrastructure

Adrenocortical and granulosa cells treated with OKA were followed ultrastructurally for varying time intervals. Adrenocortical cells were cultured for 24 h in medium containing ACTH + HDL afterwhich OKA (50 nM) was added for 15, 30, 90 min and 3, 6, and 24 h. At the electron microscope level, all control (non-treated) cells had well-developed Golgi complexes with stacked membrane cisternae, budding vesicles, free vesicles, vacuoles, and microtubules (**Fig. 2A**). By 90 min, some cells showed changes that were seen in the majority of cells at 3 h; i.e., the cells had lost their Golgi membrane stacks and retained only a tight collection of vesicles in the areas which had previously been Golgi regions (Fig. 2B). Most of the remaining vesicles did not have clathrin type coats (36, 37), but were of uniform small size (55–60 nm), and stained immunofluorescently with antisera of two different Golgi-associated proteins (58 and 110 kDa proteins). The 110 kDa protein (βCOP) has been identified as one of a complex of coat proteins (24, 35) associated with anterograde-bound Golgi vesicles and Golgi membranes (36, 37) (**Fig. 3A**), and as culture time with OKA increases, the early collection of vesicles in the Golgi area (Fig. 3B) disintegrates and is largely lost (Fig. 3C).

Whereas the most prominent OKA-induced structural change in both adrenocortical and granulosa cells was the disintegration of Golgi complexes, it was also clear that few microtubules survived OKA treatment (**Figs. 4A, 4B**). Quantitative studies with OKA-treated adrenal and granulosa cells show that, in regions retaining vesicles or other identifiable Golgi structures, microtubule content is reduced ~85% ($P < 0.001$); i.e., mean microtubule length/unit Golgi cytoplasm volume decreases from 0.242 to 0.035 μm microtubule per μm³ Golgi cytoplasm. This

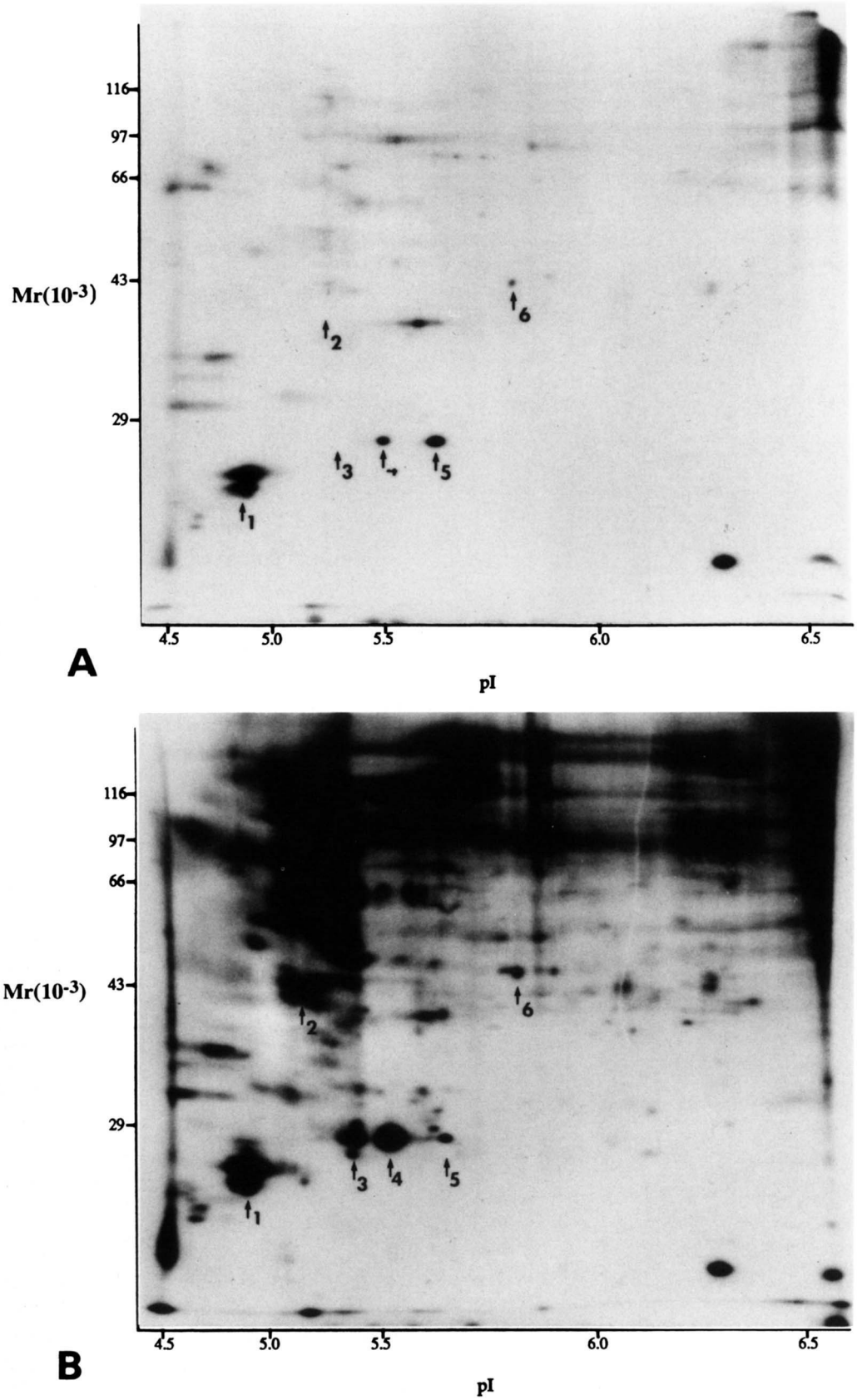


Fig. 1. Two-dimensional autoradiograms of ^{32}P-labeled adrenal cell homogenates pretreated without (A) or with OKA (B). Arrows 1–4 and 6 point to well-resolved low molecular weight proteins that show a large increase in phosphorylation following OKA treatment, whereas arrow 5 shows a loss in phosphorylation in the same autoradiogram. In each case, the concentration of protein used was 25 μg.

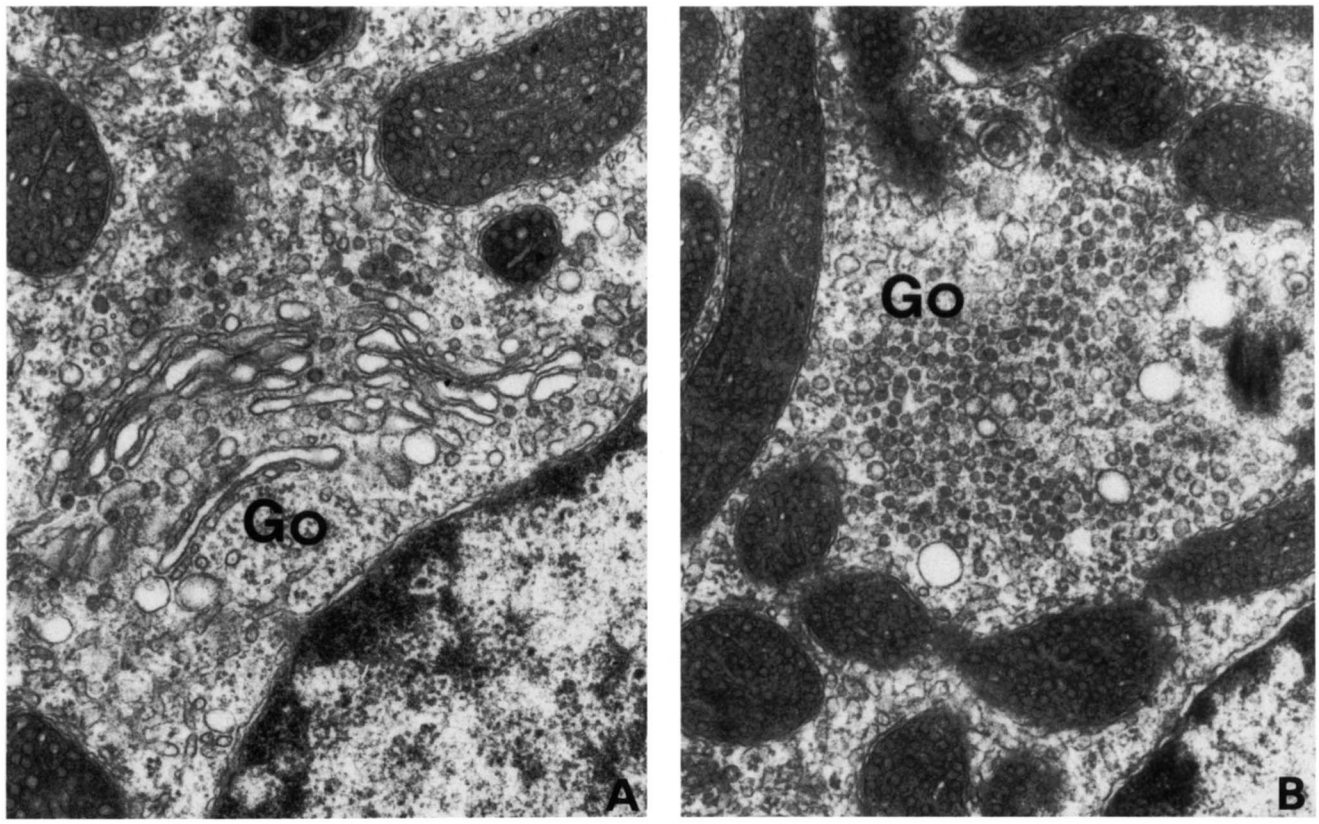


Fig. 2. Electron micrographs of Golgi regions from control (A) and OKA (50 nM, 3 h) -treated adrenal cells (B). Control adrenocortical cells have conventional Golgi complexes including stacked cisternal membranes and a variety of vesicles and vacuoles. In OKA-treated cells, one or more tight bundles of uniformly small vesicles (55–60 nm) characteristically replace the Golgi apparatus.

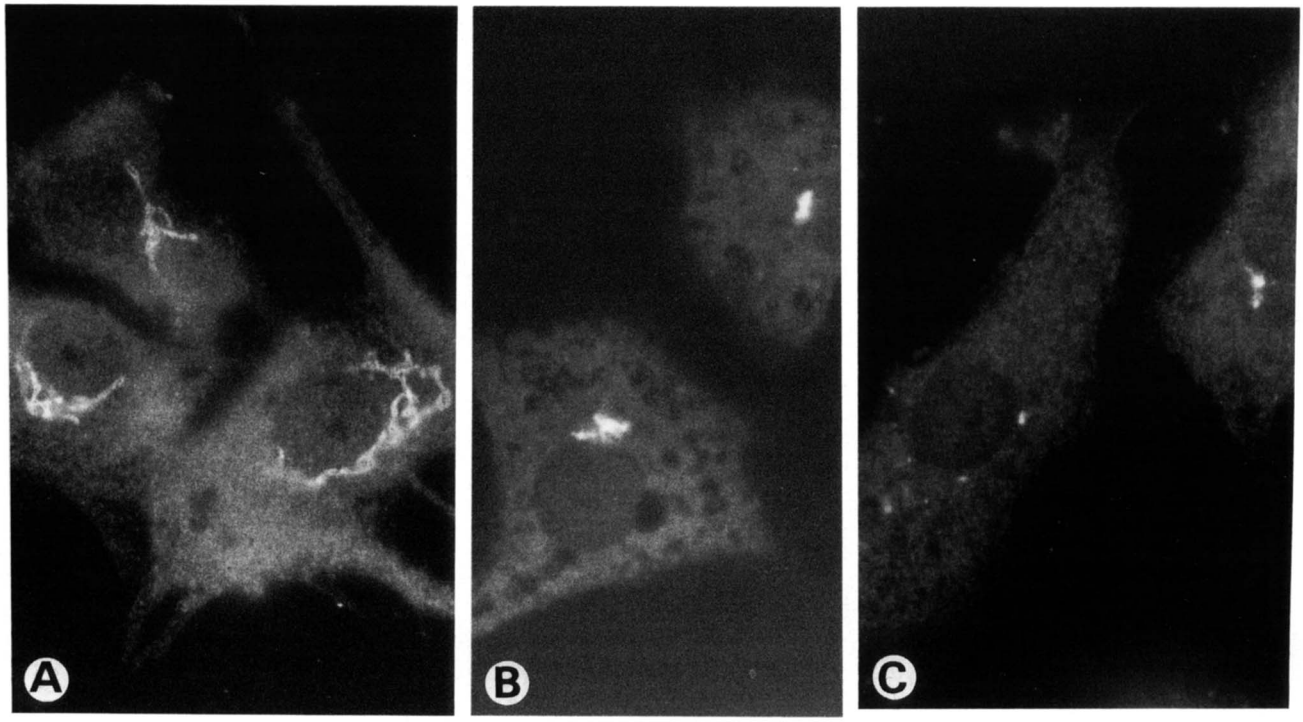
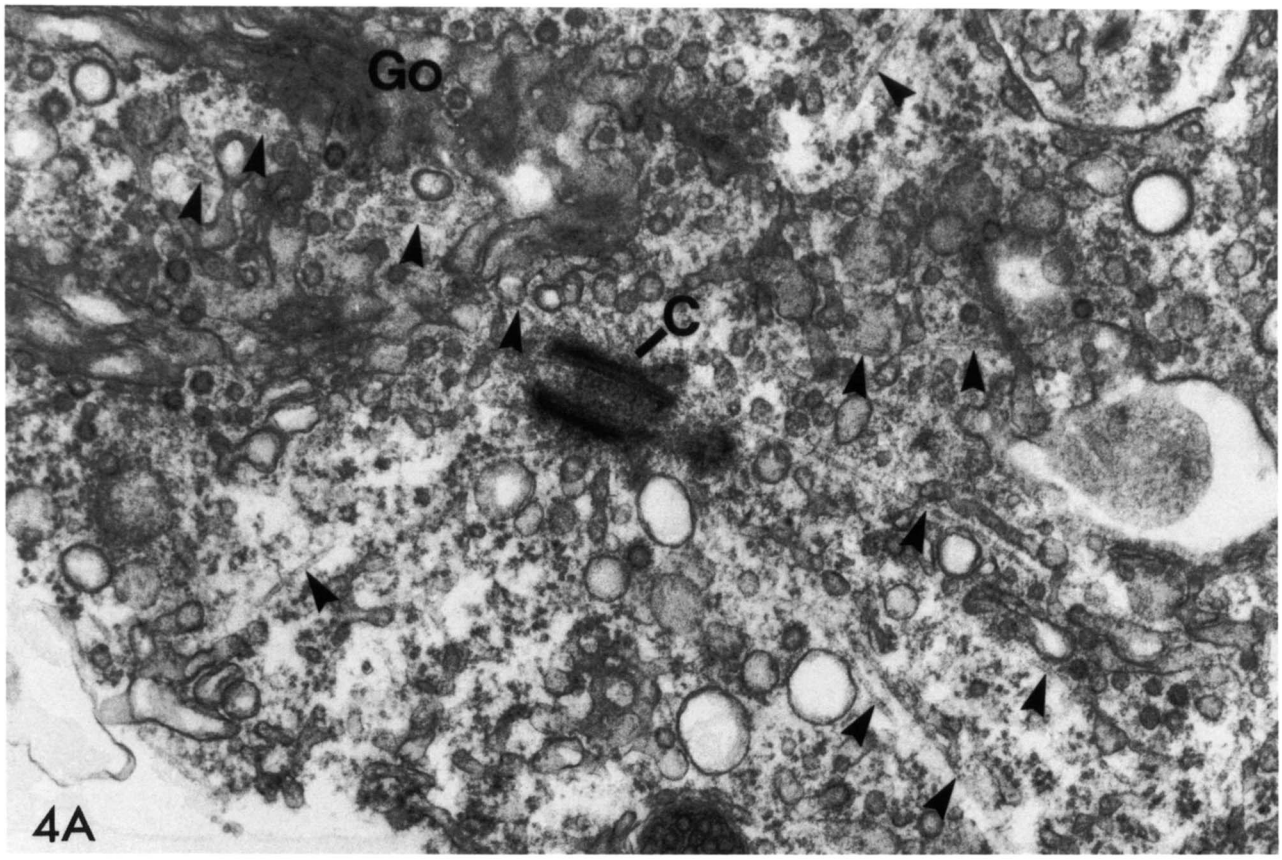


Fig. 3. Adrenocortical cells stained fluorescently with antiserum to 110 kDa Golgi-associated protein, βCOP. In control cells (A), the Golgi appears as twisted bands of fluorescence surrounding the nucleus. In 3 h OKA-treated cells (B), there are bright perinuclear spots of fluorescence, and in 6–24 h OKA-treated cells, either no fluorescence is observed or there are small dots of fluorescence as in C.



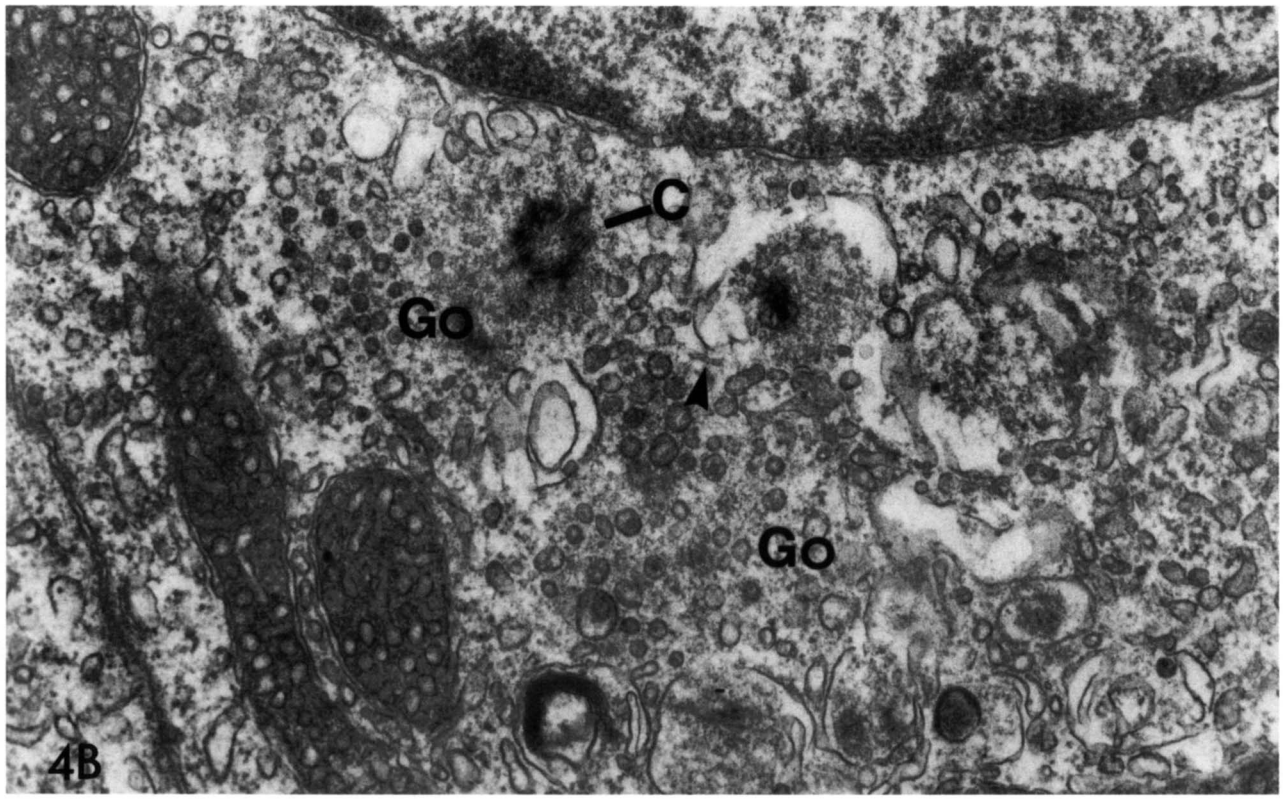


Fig. 4. Electron micrograph showing microtubules associated with centrioles in Golgi (Go) regions of control (A) and OKA-treated (B) cells. Cells were given OKA for 3 h (50 nM). In the control cell there are a large number of microtubules (arrowheads) streaming from the longitudinally sectioned centriole (C). In the OKA-treated cell, there is a cross section of a centriole (C), a clustering of small Golgi vesicles (Go) as in Fig. 2, but only one small segment of a microtubule (arrowhead).

OKA-related decrease in microtubule content did not seem to precede the breakdown of Golgi membranes as the occasional cell retaining intact Golgi cisternal membranes generally had a full complement of Golgi-associated microtubules. Likewise, in OKA reversal experiments, microtubules and Golgi membranes reappear more or less simultaneously; i.e., in cell samples obtained in frequent intervals from 15 min to 6 h after the removal of OKA, microtubules were generally not present except in cells in which there were visible reestablished Golgi cisternal membranes.

Other cell organelles (mitochondria, endoplasmic reticulum, lysosome, vesicle, vacuole, cytoskeletal filament structure, content and distribution) appeared unaffected by the same OKA conditions that resulted in the distinctive breakdown of Golgi complexes. However, 2-fold higher doses or longer time intervals (6–24 h) with OKA not only increased cell rounding and detachment, but the remaining cells showed progressive signs of poor health, i.e., increased vacuolization, reduced cytoplasmic content of mitochondria, etc.

Brefeldin A (1 μM, 3 h) treatment of adrenocortical and granulosa cells was associated with a total loss of Golgi-cisternal membranes and associated structures though in other respects the cells appeared healthy. Sampling at earlier time points (5–30 min) revealed cells with some patches of Golgi-associated vesicles, but the vesicles in these clusters were not as abundant or as characteristically uniform as after OKA treatment.

Steroidogenesis and cholesterol transport

Figs. 5A and 5B indicate that OKA (100 nM) decreases adrenocortical cell corticosterone production or granulosa cell progestin formation in the presence of stimulating hormones and HDL_3; i.e., corticosterone secretion slows by the 3-h time point, and reaches a plateau after 6 h ~90% reduced as compared to non-OKA-treated cells.

This OKA-induced decrease in cell response is not the result of reduced hormone receptor efficacy (3). Indeed, the fact that adrenocortical cells are capable of normal corticosterone production when freely diffusible 20α-

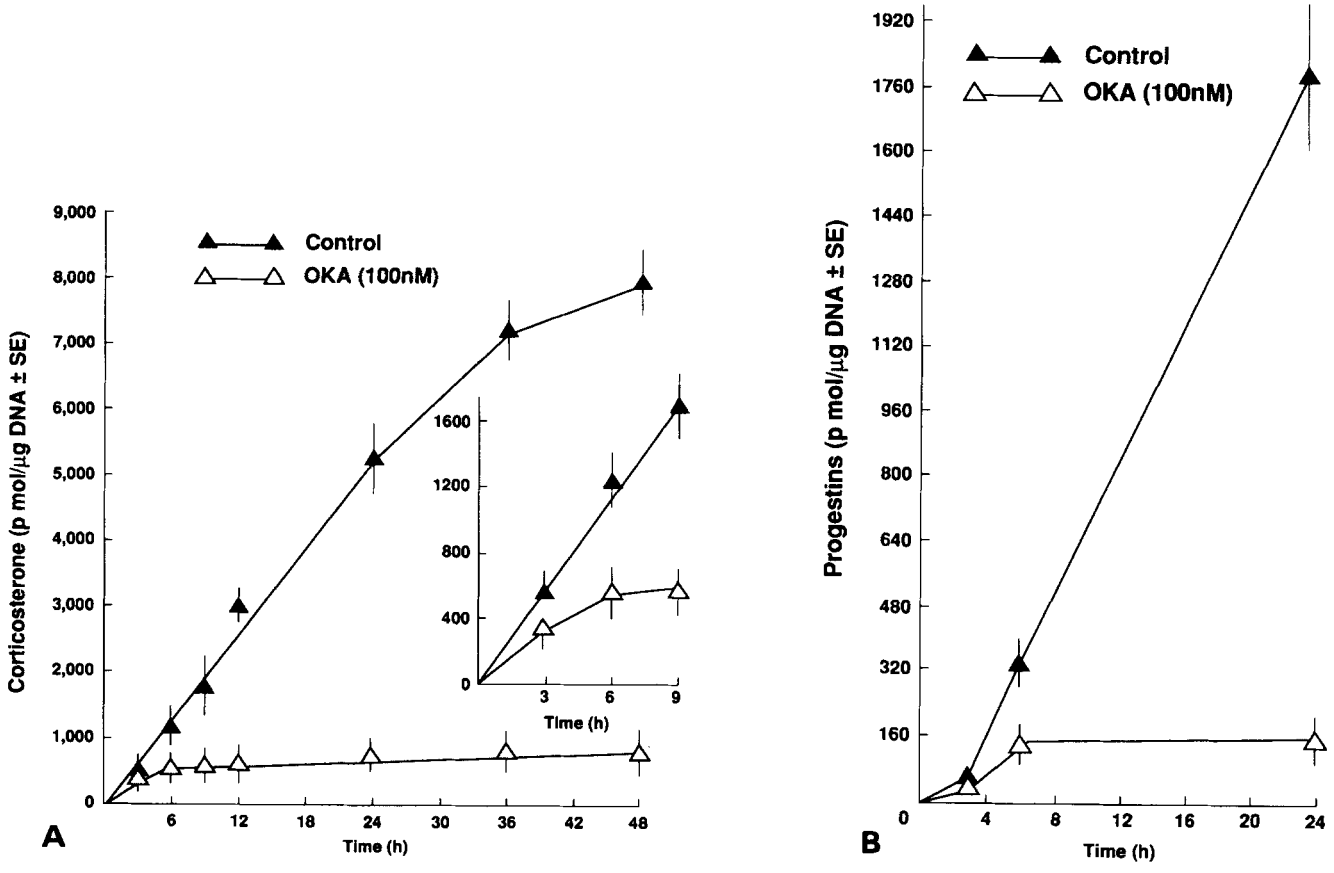


Fig. 5. Effect of time and OKA on lipoprotein-supported steroidogenesis in adrenocortical (A) and granulosa (B) cells. In both cell types, OKA has the effect of slowing hormone production by 3 h with no increase in hormone production beyond 6 h (B). In contrast, control cells slow a linear increase in production with time; by 24 h, control cells make ~10-fold the hormone made by OKA-treated cells. The insert to this figure shows an enlarged version of early time points.

hydroxycholesterol is the steroid precursor (4–6) suggests that the steroidogenic pathway itself is unaffected and that the problem observed in Fig. 5 lies in decreased utilization of lipoprotein-derived cholesterol. Data from **Table 3** suggest that this is correct and that, in intact adrenocortical cells pretreated with OKA (100 nM) for 3–6 h, the total mass of corticosterone synthesized and the proportion of newly available radiolabeled cholesteryl oleate converted to corticosterone is significantly reduced regardless of which species of lipoprotein is used as the cholesterol donor. Thus, at these early time points, ~15–30% of the cholesteryl ester utilized by the cells comes from newly acquired (labeled) cholesterol, and the conversion of this cholesterol to corticosterone is between 80 and 90% reduced after treatment with OKA for 3–6 h. That inefficient cholesteryl ester hydrolysis (from intracellular lipid storage sites) is not the source of the problem is suggested by the fact that cholesteryl esterase activity itself does not appear to be OKA-sensitive; i.e., when adrenocortical cell cytosol is incubated with OKA (0–1000 nM) no changes in enzyme activity as compared to untreated cytosol can be measured (data not shown).

The information in **Table 4** addresses the question of whether the OKA-induced defect in cholesterol conversion to steroid hormone is a function of an OKA-induced change in internalization of lipoprotein-cholesterol. Adrenocortical cells were treated with OKA and, at the same time, provided with lipoproteins designed to utilize either the endocytic (hLDL, rHDL) or selective (rHDL, $hHDL_3$) pathway in delivering cholesteryl esters (12, 13). Five separate studies were carried out using the three different preparations of lipoproteins. Although there was internal consistency within individual studies, labeling variations among the different preparations of lipoproteins resulted in cumulative data with large standard errors. Despite this, the effect of OKA on cholesteryl ester uptake is clear; i.e., through 6 h of incubation, OKA shows no effect on the internalization of cholesteryl esters by either of the two cholesteryl ester pathways. On the other hand, by 24 h of OKA treatment, the total uptake of cholesteryl ester is significantly reduced regardless of which lipoprotein is the donor and which cholesterol pathway is utilized.

Since the OKA-induced decrease in cholesteryl ester conversion (Table 3) and steroid hormone production (Fig. 5) occurred several hours before there was a detectable problem in lipoprotein cholesteryl ester uptake by the cells (Table 4), it was likely that cholesterol was available to the cell, but through some action of OKA was not being appropriately transported to key side-chain cleavage sites in mitochondria. The cause could be OKA-induced problems in transport of cholesterol to mitochondria, or problems in cholesterol transport across mitochondrial membranes, but was unlikely to involve the mitochondrial-located P450scc enzyme itself insofar as the same cells were able to efficiently produce corticosterone from 20α-hydroxycholesterol (4–6).

To address the first possibility, we isolated mitochondria from adrenal cells that had been treated with, or without, OKA (100 nM) for 30 min and then incubated for 3 h with cholesterol oleate-labeled lipoproteins [50 μg/ml $hHDL_3$, tritium-label in the cholesterol moiety]. Our

TABLE 3. Effect of okadaic acid on the conversion of lipoprotein-derived [^{3}H]cholesteryl oleate into corticosterone

Lipoprotein (100 μg protein per ml)	OKA (100 nM)	[^{3}H]Cholesteryl Oleate Incorporated into Corticosterone	Mass of Corticosterone Synthesized
		pmol/μg DNA ± SE	*pmol/μg DNA ± SE*
Incubation time 3 h			
[^{3}H]CO-$hHDL_3$	–	174 ± 10	651 ± 108
	+	32 ± 4	310 ± 39
[^{3}H]CO-rHDL	–	198 ± 32	655 ± 84
	+	41 ± 9	285 ± 46
[^{3}H]CO-hLDL	–	202 ± 28	590 ± 85
	+	40 ± 9	350 ± 33
Incubation time 6 h			
[^{3}H]CO-$hHDL_3$	–	497 ± 50	1460 ± 129
	+	57 ± 12	544 ± 71
[^{3}H]CO-rHDL	–	466 ± 83	1378 ± 144
	+	50 ± 14	678 ± 107
[^{3}H]CO-hLDL	–	450 ± 50	1406 ± 182
	+	62 ± 14	468 ± 96

Cultured adrenocortical cells were incubated with [^{3}H]CO-labeled lipoproteins + ACTH (10 ng/ml) ± OKA (100 nM) for 3 or 6 h. [^{3}H]corticosterone synthesis (i.e., [^{3}H]CO incorporation into corticosterone was measured by TLC. The mass of corticosterone produced was quantified fluorometrically. Results are mean ± SE of four separate experiments; CO, cholesteryl oleate.

TABLE 4. Effect of okadaic acid on the uptake (internalization) of lipoprotein-derived cholesteryl esters

Lipoprotein	Okadaic Acid (100 nM)	Total Mass of of Cholesteryl Ester Internalized	Mass of Cholesteryl Ester Internalized via the Endocytic Pathway	Mass of Cholesteryl Ester Internalized via the Selective Pathway
3-h Incubation				
$hHDL_3$	–	1231 ± 308	53.4 ± 14.3	1179 ± 312
	+	1412 ± 367	62.1 ± 17.8	1349 ± 381
rHDL	–	1657 ± 528	242 ± 22.4	1293 ± 608
	+	1355 ± 530	300 ± 23.9	1109 ± 546
hLDL	–	1418 ± 548	640 ± 170	982 ± 502
	+	1405 ± 434	578 ± 184	827 ± 352
6-h Incubation				
$hHDL_3$	–	1694 ± 263	73.1 ± 13.6	1520 ± 261
	+	1637 ± 221	71.5 ± 27.9	1566 ± 264
rHDL	–	1416 ± 370	509 ± 169	1152 ± 373
	+	1349 ± 455	269 ± 45	1079 ± 503
hLDL	–	1615 ± 393	878 ± 269	805 ± 193
	+	1497 ± 572	845 ± 334	652 ± 239
24-h Incubation				
$hHDL_3$	–	4356 ± 657	71.1 ± 19.0	4276 ± 642
	+	1575 ± 534	86.2 ± 16.3	1491 ± 513
rHDL	–	3043 ± 561	573 ± 84	2469 ± 639
	+	846 ± 345	446 ± 152	952 ± 505
hLDL	–	3873 ± 500	2279 ± 736	1597 ± 540
	+	2002 ± 939	1302 ± 700	632 ± 248

Results are expressed as pmole cholesteryl ester internalized/μg DNA ± SE (n ' 5). Protein/cholesterol ratios of $hHDL_3$ = 2.42; rHDL = 1.1; hLDL = 0.55.

measurements showed that incubated mitochondria from OKA-treated and control cells had accumulated similar amounts of [^{3}H]cholesterol (data not shown). However, since mitochondrial fractions isolated from adrenocortical cells are not entirely free of contamination with endoplasmic reticulum (E. Reaven, unpublished observation), which could sequester cholesterol, it was necessary to see whether the newly acquired mitochondrial cholesterol had, in fact, reached the P450scc sites. For these experiments, adrenocortical cells with equally plentiful endogenous stores of cholesterol [i.e., cells taken from control (non-4APP) rats] were treated with ACTH ± OKA (100 nM) as above, after which their mitochondria were isolated and tested for in vitro pregnenolone production. **Fig. 6A** indicates that under these conditions mitochondria from OKA-treated cells are capable of producing only 50% of the pregnenolone produced by mitochondria from non-OKA-treated cells, suggesting that in the OKA-treated cells, sufficient cholesterol had not arrived at the working sites of cholesterol side chain cleavage enzymes. In Fig. 6B, mitochondria from control or OKA cells incubated with 20α-hydroxycholesterol show no differences in pregnenolone production.

DISCUSSION

Earlier studies from our group suggested that the observed OKA effect on steroidogenesis is due to the lack of appropriate cholesterol substrate for steroidogenesis (3), and initially it was believed that exogenous, lipoprotein-derived cholesteryl esters could not be efficiently internalized by cells after OKA treatment (3). However, the current studies with adrenocortical and granulosa cells indicate that internalization of cholesteryl esters is not the main problem; i.e., at early time points when experiments can demonstrate decreased steroidogenesis and decreased conversion of newly acquired cholesterol to pregnenolone, no deficiencies in lipoprotein cholesteryl ester uptake can be measured.

How then does OKA affect steroidogenesis? It is well understood that OKA has the capacity to influence the phosphorylation of many cellular proteins (2) and our own 2-dimensional gels confirm the fact that a number of adrenal cell proteins are superphosphorylated after OKA treatment. Presumably this increase in protein phosphorylation occurs because OKA inhibits the functioning of serine/threonine phosphatases of class 1 and 2A (1, 2); the affected proteins are unable to dephosphorylate and are consequently trapped in a hyperphosphorylated state. As we know that a number of adrenal steroidogenic proteins rapidly phosphorylate in response to trophic hormone stimulation (38, 39), it might be expected that OKA influences these proteins directly. However, our studies show that the functions of the steroidogenic enzymes are not themselves affected. If, for example, cholesterol is available to adrenal cell mitochondria through the use of freely diffusible substrates such as 20α-

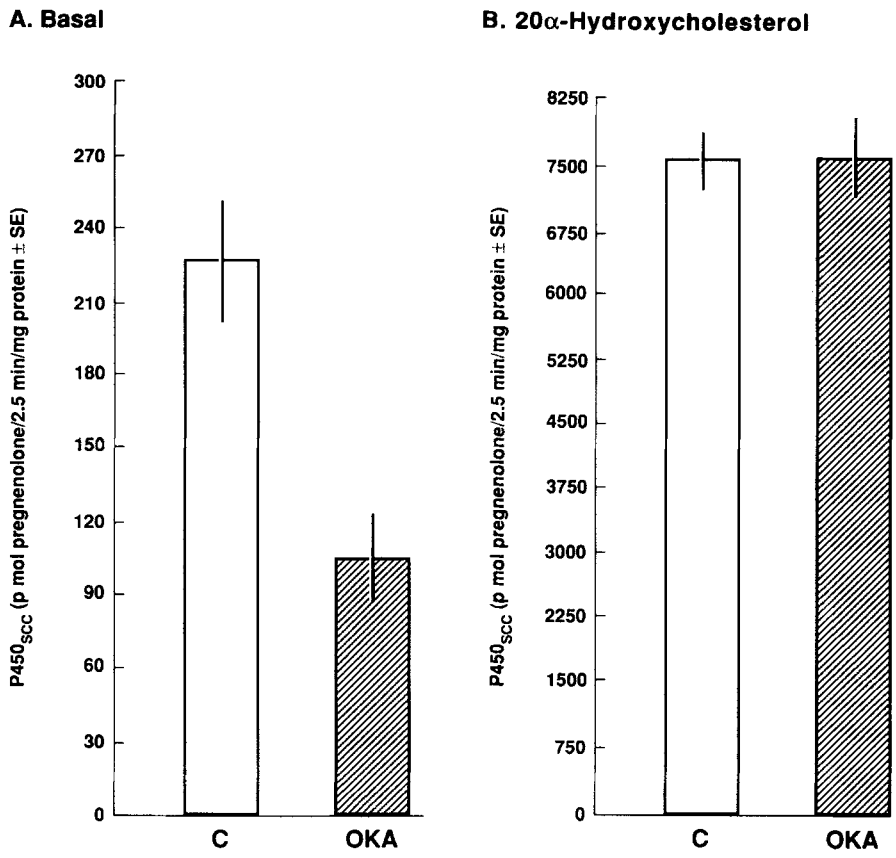


Fig. 6. Cholesterol side-chain cleavage (P450scc) activity in mitochondria of control and OKA-treated cells. Adrenocortical cells containing ample stores of cholesterol (obtained from non-4APP-treated rats) were incubated with ACTH ± OKA. In A, mitochondria were isolated and used directly for the measurement of cholesterol side chain cleavage (i.e., the conversion of free cholesterol to pregnenolone by mitochondrial P450scc). Mitochondria from OKA-treated cells converted 50% less cholesterol to pregnenolone than control cells, suggesting that less cholesterol was available at the mitochondrial P450scc sites. In B, mitochondria from the same preparations were incubated with a freely diffusible form of exogenous cholesterol (20α-hydroxycholesterol). Under this condition, the mitochondria from OKA-treated cells and control cells converted equal amounts of cholesterol to pregnenolone, suggesting that OKA does not interfere with P450scc activity per se.

hydroxycholesterol, steroidogenesis continues normally, even in the presence of OKA (4–6).

On the other hand, our results show dramatically that with OKA treatment, new cholesteryl esters acquired by the cells (e.g., lipoprotein-derived [^{3}H]cholesteryl oleates) are not efficiently converted to hormone; indeed, cholesteryl oleate conversion to corticosterone is reduced by ~80% at 3 h and ~90% by 6 h in OKA-treated cells. In addition, isolated mitochondria from OKA-treated cells have reduced ability to mobilize cellular cholesteryl ester stores for pregnenolone production. As mitochondrial steroidogenic enzymes are not directly sensitive to OKA, how is it that mitochondria are affected? There are a number of plausible answers which relate to the availability of precursor cholesterol at key intracellular organelle (i.e., P450scc) sites (36, 40, 41). For one, cholesterol movement from the cell cytoplasmic to the outer mitochondrial membrane may be blocked by OKA. Mitochondrial preparations from cultured adrenal cells are often contaminated with fragments of endoplasmic reticulum and, as such, some cholesterol which had been sequestered there (and measured with the mitochondrial fraction) may not actually have reached the mitochondria. Even if cholesterol had reached the mitochondria, OKA treatment may have prevented its traversing the outer membrane to reach a variety of intramitochondrial cholesterol pools (40) including the P450scc sites (41). As various tissue factors have been identified and investigated (42–50) in steroidogenic cells, the distinction between cytoplasmic sterol carrier proteins and mitochondrial transfer proteins has become blurred. All of these factors promote the utilization of free cholesterol by mitochondria, but their mechanism of action appears to differ. The most likely candidate for a sterol-binding protein actually translocated in the cytoplasm is sterol carrier protein$_2$ (SCP$_2$). This protein is known to stimulate a number of

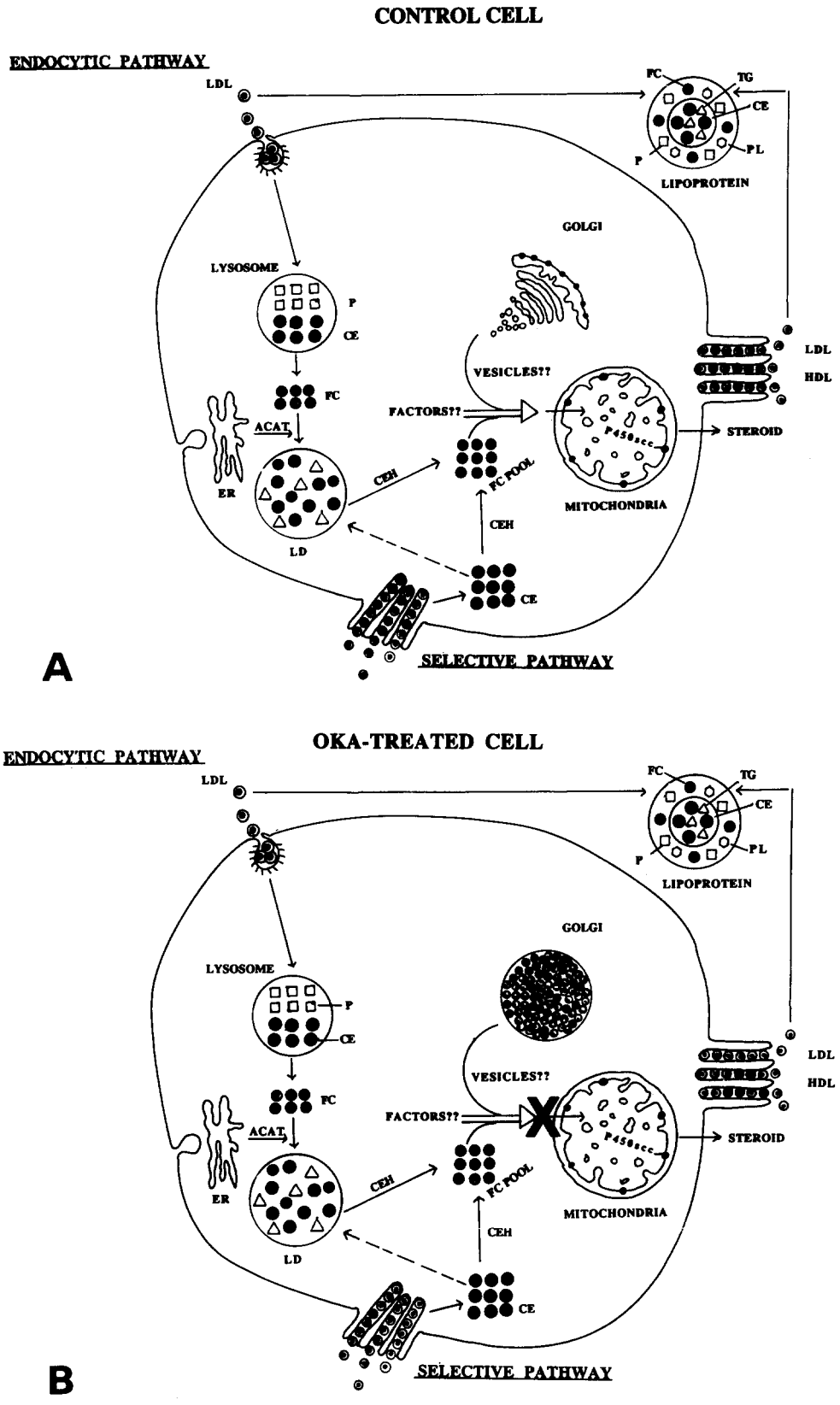


Fig. 7. Diagram of uptake, processing, and utilization of lipoprotein-derived cholesterol for steroid hormone production in control cell (A) and OKA-treated cell (B). X, failed transport of free cholesterol (FC) to mitochondrial P450scc sites; CE, cholesteryl ester; CEH, cholesteryl ester hydrolase; P, protein; TG, triglyceride; PL, phospholipid; ER, endoplasmic reticulum; ACAT, acyl CoA:cholesterol acyltransferase; LD, lipid droplets.

cholesterol-related reactions, presumably by transporting cholesterol or its metabolites (or a variety of other lipids) to relevant enzymes (44, 46–49, 51). Although the mechanism by which SCP_2 stimulates side-chain cleavage activity in adrenal mitochondrial is unknown, it has been suggested that it serves as a conduit for cholesterol from inaccessible to accessible sites on the mitochondrial outer membrane (44, 46–49, 51). As such, it differs from other cycloheximide-sensitive "labile" protein mediators within mitochondria which appear to more directly regulate intramitochondrial cholesterol movement. These factors are steroidogenic activator polypeptide [SAP (45)], guanosine triphosphate [GTP (47)], and the recently described diazepam binding inhibitor [DBI (50)]. Whereas all of these factors are isolated from the adrenal and reportedly function at the rate-limiting step of translocation of cholesterol to the inner membrane where side-chain cleavage to pregnenolone occurs (41), only SCP_2 is known to be a phosphorylated protein. As such, SCP_2 could be a prime target for the action of OKA.

Other mechanisms for cholesterol trafficking may be used by steroidogenic cells. Various model systems have shown lipid components such as plasma membrane sphingomyelins (SM) have a high affinity for cholesterol (52, 53). It is now well accepted that a SM-vesicle recycling pathway exists in cells (48–50), that SM biosynthesis occurs in the Golgi compartment, and that SM is transferred by Golgi vesicles to the plasma membrane (54–56) after which SM-plasma membrane-vesicle internalization occurs. Cholesterol deprivation apparently interferes with this process (57). Recent studies with apoE-HDL complexes propose a more direct involvement of the Golgi compartment in the 'selective' uptake of cholesteryl esters, i.e., internalization of apoE-HDL complexes by cells followed by delivery of these complexes to the trans-Golgi network where depletion of CE occurs (58). It may be that OKA compromises processes dependent on Golgi-associated vesicle synthesis and/or cycling (30) and, as a result, cholesterol transport (along with the transport of a variety of other Golgi-mediated products) fails. It is of interest that some of the most prominent OKA-hyperphosphorylated proteins visualized by our 2D gel system are in the 20–35 kDa size range where a number of endosome/Golgi-specific phosphoproteins have been identified (59).

It is of interest, too, that other compounds that cause the breakdown of Golgi membranes also interfere with steroidogenesis. This is the case with agents such as colchicine (60–62) which target microtubule proteins (63), and with brefeldin A (see Table 2) which targets Golgi membrane coat proteins (36, 64–66). Ultimately all these agents cause the disintegration of the Golgi complex (see discussion in ref. 30), but presumably they do so by different primary mechanisms.

Thus, the challenge for the future will be to identify those specific phosphoproteins that OKA targets in steroidogenic cells, and to determine their intracellular sites of action and their link to processes affecting the intracellular transport of cholesterol. **Fig. 7A** attempts to diagram the important events in the uptake, processing, and utilization of lipoprotein-mediated cholesterol for steroid hormone production. The diagram of Fig. 7B focuses on the observation that, while most of the cell appears morphologically intact and functions normally in OKA-treated cells, the Golgi compartment is destroyed. We suggest that in some way this event may account for the retarded arrival of cholesterol at mitochondrial side-chain cleavage sites.

This research was supported by the National Institutes of Health grant HL-33881.

Manuscript received 2 July 1993 and in revised form 10 January 1994.

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